Molecular mechanisms of protein sorting in the synaptic vesicle life cycle.

Thesis

UNRESTRICTED

Dario Bonanomi

MOLECULAR MECHANISMS OF PROTEIN SORTING IN THE SYNAPTIC VESICLE LIFE CYCLE

Thesis submitted in partial fulfilment of the requirements of the Open University for the Degree of Doctor of Philosophy in Molecular and Cellular Biology

August 2005

DIBIT-Department of Biological and Technological Research
San Raffaele Scientific Institute
Milan, Italy

DATE OF SUBMISSION 1 AUGUST 2005
DATE OF AWARD 30 JANUARY 2006

CONTENTS

DECLARATION

This Thesis has been written by myself and has not been used in any previous application for a degree. All the Results presented were obtained by myself, except for the experiment shown in Figure 3.8 A, which has been performed by Dr. Fabio Benfenati (University of Genova, Italy). All sources of information are acknowledged by means of reference.
Some of the work contained in this Thesis has been published:

M. Pennuto*, **D. Bonanomi***, F. Benfenati, F. Valtorta (2003). Synaptophysin I controls the targeting of VAMP2/synaptobrevin II to synaptic vesicles.
Mol Biol Cell. 14:4909-4919. [* Equal contribution]

F. Valtorta, M. Pennuto, **D. Bonanomi**, F. Benfenati (2004). Synaptophysin: leading actor or walk-on role in synaptic vesicle exocytosis?
BioEssays. 26:445-453. (Rewiew)

D. Bonanomi, M. Pennuto, M. Rigoni, O. Rossetto, C. Montecucco, F. Valtorta (2005). Taipoxin induces synaptic vesicle exocytosis and disrupts the interaction of synaptophysin I with VAMP2.
Mol Pharmacol. 67:1901-1908.

D. Bonanomi, A. Menegon, A. Miccio, G. Ferrari, A. Corradi, H.-T. Kao, F. Benfenati, F. Valtorta (2005). Phosphorylation of synapsin I by cyclic AMP-dependent protein kinase controls synaptic vesicle dynamics in developing neurons.
J Neurosci. 25: 7299-7308.

ABSTRACT

Synaptic vesicle (SV) proteins are synthesized at the level of the cell body and transported along the axons in precursor vesicles which undergo cycles of exo-endocytosis in transit to either the distal growth cone or the nerve terminal. I sought to investigate the mechanisms underlying the sorting of SV components and to determine whether and how SV exocytosis is regulated prior to synaptogenesis.

SV fusion is underlain by the interaction of vesicle-associated membrane protein (VAMP) 2 with plasma membrane partners. Fluorescence resonance energy transfer analysis reveals that VAMP2 interacts with Synaptophysin I (SypI), a major resident of SVs, on the SV membrane and that exocytotic stimuli cause dissociation of this complex at a stage preceding SV fusion. This observation is consistent with the idea that SypI limits VAMP2 availability for fusogenic complexes. The interaction between SypI and VAMP2 occurs early along the exocytotic pathway and is required in order for SypI to govern the sorting of VAMP2 to SVs. The control of VAMP2 sorting by its negative regulator SypI establishes a mechanism which prevents the fusion at inappropriate sites of SVs directed to the nerve terminal.

The study of SV dynamics in developing hippocampal neurons reveals that cAMP-dependent pathways affect SV distribution and recycling in the axonal growth cone and that these effects are mediated by the SV-associated phosphoprotein synapsin I. Synapsin I and its phosphorylation by cAMP-dependent protein kinase A play a pivotal role in regulating SV organization and dynamics in neuronal growth cones and in determining the formation of synaptic contacts. These results provide new clues as to the bases of the well known activity of synapsin I in synapse maturation and indicate that molecular mechanisms similar to those operating at mature nerve terminals are active in developing neurons to regulate the SV life cycle prior to synaptogenesis.

INTRODUCTION

1.1 MOLECULAR ASPECTS OF SYNAPTIC VESICLE BIOGENESIS

Synaptic vesicles (SVs) are characterized by a specific complement of proteins which govern their traffic in neurons. Many of these proteins have been studied in detail using a variety of experimental approaches and linked to at least putative functions in the regulation of SV exo-endocytosis (Sudhof, 2004). These studies, together with the physiological description of SV trafficking in the presynaptic terminal, make the SV by far the best characterized organelle of eukaryotic cells. Thus, the SV cycle has served as one of the paradigms for understanding the molecular basis of vesicular traffic in the eukaryotic cell. Three main aspects of the SV life cycle can be distinguished: a) the *de novo* biogenesis of SVs, b) fusion of SVs with the presynaptic plasma membrane, and c) recycling of the SV membrane after exocytosis for the re-formation of SVs. While the latter two processes have been extensively studied and are relatively well understood, comparatively little is known about the molecular events underlying the *de novo* biogenesis of SVs. Because of the need for experimental approaches designed at unravelling the biosynthetic pathways of SV proteins, the isolated nerve terminal preparations (synaptosomes), that have been widely exploited in the study of SV exocytosis and recycling, are not useful to explore SV biogenesis. Moreover, such studies are technically demanding in the case of primary neuronal cultures because of the number of cells required. It is for these reasons that studies addressing the biogenesis of SVs have been mostly carried out with neuroendocrine cell lines.

Neuroendocrine cells contain vesicles that are highly related to neuronal SVs of neurons and are therefore referred to as synaptic-like microvesicles (SLMVs). These organelles contain several integral membrane proteins that are typical of SVs (Clift-O'Grady et al., 1990), exhibit a small and uniform size comparable to the size of SVs (Cameron et al, 1991; Linstedt and Kelly, 1991b), and are capable of taking up, storing and releasing neurotransmitters (Bauerfeind et al., 1993 and 1995). Despite these similarities, it is unclear whether SLMVs, as SVs, undergo exocytosis upon stimulation in a calcium-dependent fashion. Indeed, it seems that SLMVs are consumed by constitutive fusion with the plasma membrane (Faundez et al., 1997, but see Bauerfeind et al., 1995). These differences in the exocytotic behaviour between SLMVs and SVs may have important implications for the membrane traffic events which regulate the biogenesis of the two types of vesicles. Moreover, neuroendocrine cells do not form axons and lack synapses and synaptic specializations. Thus, it seems unlikely that the same trafficking routes are undertaken by proteins destined to SVs and SLMVs in neurons and neuroendocrine cells, respectively. These premises are of particular relevance since most of the current knowledge about the molecular events underlying the formation of SVs has been extrapolated from the study of the biogenesis of the SLMVs of the neuroendocrine rat pheochromocytoma-derived line PC12.

1.1.1 Biogenesis of SLMVs from the endocytotic pathway

The endocytotic origin of SLMVs has been known for some time. SLMVs in PC12 cells take up the exogenous phase marker horseradish peroxidase (HRP), and following surface labelling and subsequent warming, labelled proteins become incorporated into SLMVs (Johnston et al., 1989; Clift-O'Grady et al., 1990; Bauerfeind et 1993). Synaptophysin, a major membrane protein of SVs (Wiedenmann and Franke, 1985; Jahn et al., 1985) and SLMVs (Navone et al., 1986) shows the intrinsic tendency to

accumulate in transferrin receptor-containing endosomes in both PC12 cells and transfected fibroblasts (Johnston et al., 1989; Cameron et al., 1991; Linstedt and Kelly, 1991b). These findings suggest that the donor membrane for the biogenesis of SLMVs is either the plasma membrane, or the recycling endosome, or both.

It is important to remember that in either case the formation of SLMVs implies a sorting step that segregates the SLMV membrane proteins from the normal residents of the plasma membrane or endosomes. Consistently, transferrin receptor is excluded from SLMVs generated by endocytosis (Clift-O'Grady et al., 1990).

1.1.2 Formation of SLMVs from early endosomes

Several lines of evidence point to the involvement of an intracellular compartment, most likely an endosomal intermediate, in the formation and reformation of SLMVs. The fluid phase marker HRP is detected in transferrin receptor-positive endosomes and not in SLMVs after a pulse internalization (5 min) and short chase (7 min), but appears in the latter organelles after a longer chase (3 h; Bauerfeind et al., 1993). This indicates that SLMVs can be generated by budding from endosomal compartments, albeit in a process requiring > 7 min. Pulse-chase labelling was used to investigate the exit of newly synthesized synaptophysin from the trans-Golgi network (TGN) and its transport to its final destination, the SLMVs (Régnier-Vigoroux et al., 1991). This study allowed to propose a model for SLMV biogenesis in neuroendocrine cells according to which after synthesis in the rough endoplasmic reticulum and passage through the Golgi complex, integral membrane proteins destined to SLMVs first move from the TGN to the plasma membrane in constitutive secretory vesicles. They then cycle several times between the plasma membrane and endosomes and eventually segregate from resident proteins prior to being incorporated into SLMVs budded from endosomes. It is unclear

whether, in addition to synaptophysin, other SLMV components undertake this routing pathway and to what extent this model can be applied to SV biogenesis in neurons.

The usefulness of PC12 cells as a model was furthered by the development of a cell-free system for SLMV biogenesis (Desnos et al., 1995). In this assay, PC12 cells placed at 15 °C accumulate internalized antibodies against an epitope-tagged form of synaptobrevin II/VAMP2, an integral protein of both SVs and SLMVs. Upon rewarming to 37°C, the internalized antibodies are delivered to SLMVs, suggesting that an intracellular intermediate functions as SLMV donor compartment. SLMV formation *in vitro* is time and temperature-dependent, does not require calcium but needs ATP and GTP hydrolysis (the dependency on GTP hydrolysis was also reported for the generation of endocytotic vesicles from the plasma membrane, Takei et al., 1995). Importantly, brain, but not fibroblast, cytosolic extract allows vesicles of the correct size to form, implicating a neuro-specific factor in the budding of new SLMVs from the 15°C donor compartment. SLMV reformation from the 15°C donor compartment requires the cytosolic proteins ADP ribosylation factor-1 and adaptor protein complex AP-3 (Faundez et al., 1997 and 1998), but not clathrin (Faundez et al., 1998; Shi et al., 1998). The budding step mediated by ARF1 and AP3 is from an endosomal precursor which contains internalized transferrin and occurs concomitantly with the sorting of SV proteins from other membrane protein constituents of the endosome (Lichtenstein et al., 1998).

Also another member of the ARF GTPase family, ARF6, which is highly expressed in the brain and neuroendocrine cells, and localized at both plasma membrane and endosomes, has been implicated in SLMV biogenesis. The expression of ARF6 mutants in PC12 cells affects the selective targeting of proteins to SLMVs (Powelka and Buckley, 2001). It remains to be established whether the effects reported in this study

reflect the involvement of ARF6 in vesicle formation or rather the interference of the exogenous proteins with the ARF1-mediated pathway.

A direct morphological evidence for a pathway in which SLMV membrane proteins recycle from the plasma membrane to endosomes before their incorporation into newly formed SLMVs was provided by de Wit et al. (1999) The authors exploited quantitative immunoelectron microscopy to show that although transferrin receptor and SLMV proteins, such as synaptophysin and VAMP2, exhibit a high degree of colocalization in incoming endocytic vesicles, they are largely separated after transit through early endosomes (i.e., at the level of early endosome-associated tubulovesicles which represent the domains involved in protein recycling; Klumperman et al., 1993). Moreover, by tracking the intracellular traffic of the epitope-tagged VAMP2 these authors showed that after being internalized via clathrin-coated vesicles the chimera accumulates in the vacuolar part of early endosomes at 15°C and is then redistributed to the associated tubular extensions and eventually to SLMVs after release of the 15°C block. These data strongly suggest that after endocytosis from the plasma membrane, SLMV proteins are sorted away from non-SLMV proteins at the level of early endosomes vacuoles and become selectively enriched in the tubular extensions which function as donor compartments for the budding of new SLMVs.

A primary endocytic vesicle involved in trafficking of SV proteins has been identified as part of the endocytic recycling system in neurons and PC12 (Provoda et al., 2000). In PC12 cells this vesicular compartment, which is derived by endocytosis from the plasma membrane, has been shown to contain both SV proteins (synaptophysin, synaptotagmin, SV2) and other recycling proteins (transferrin receptor, glucose transporters). Rather than representing a distinct type of regulated secretory organelles, this vesicle population is likely to correspond to the incoming endocytic vesicles

visualized by de Wit et al. (1999), in which SLMV-specific antigens and other recycling proteins are not segregated yet (but see Thoidis et al., 1998).

Endosomal sorting has also been implicated in the generation of different secretory vesicles in the nerve terminal of noradrenergic neurons, namely SVs which contain acetylcholine and large dense-core vesicles which contain noradrenaline. The constituents of both types of vesicles are internalized and recycled through a common early endosomal compartment after exocytosis (Partoens et al., 1998). This suggest that *in vivo* the postexocytic trafficking and, in particular, the ability of the cell to separate different pools of membrane proteins is of vital importance. This concept has been further strengthened by the findings obtained by Cutler's group as to the targeting of exogenous P-selectin in PC12 cells (Strasser et al., 1999). After secretagogue-stimulation P-selectin associated with dense-core granules appears on the plasma membrane and then passes through transferrin receptor-positive endosomes en route to SLMVs. Secretagogue-triggered transfer between the two classes of organelles is also observed for VAMP2 and synaptotagmin. Passage through an endosomal sorting compartment might be needed in order to effectively separate proteins destined to SLMVs from proteins that are only to be found in dense-core granules and therefore are en route back to the TGN where this class of organelles is generated (Tooze and Stinchcombe, 1992).

1.1.3 Formation of SLMVs from the plasma membrane

Schmidt et al. (1997) have described a second pathway of SLMV biogenesis which is AP-2, dynamin and clathrin dependent. The authors proposed the involvement of a novel compartment that is distinct from the transferrin receptor-containing endosome and connected to the plasma membrane via a narrow membrane continuity. The key observation, which provided the basis for the identification and characterization of the

plasmalemma-associated SLMV donor compartment, was that upon cell surface biotinylation at 18°C, a temperature which blocks the appearance of synaptophysin in SLMVs, all of the biotinylated synaptophysin is present in avidin-protected membrane, the majority of which is accessible to a reducing agent, and hence in continuity with the plasma membrane. Subplasmalemmal tubulo-cisternal compartment implicated in SLMV targeting of synaptophysin does not contain detectable levels of biotinylated transferrin receptor, indicating that the two proteins segregate at the plasma membrane. From the subplasmalemmal compartment, a minor proportion of synaptophysin (10-15%) is directly incorporated into SLMVs, whereas the majority of the protein is delivered to early endosomes, from which is then recycled back to the plasma membrane.

Using a different *in vitro* assay in which only SV proteins on the plasma membrane are labelled, the existence of a plasma membrane-derived pathway of SLMV biogenesis in PC12 cells was confirmed, albeit only as a minor pathway (Shi et al., 1998). Unlike the biogenesis of SLMVs from PC12 endosomes, the formation of plasma membrane-derived SLMVs uses the adaptor protein AP2 instead of AP3 and is BFA insensitive. Moreover, it requires clathrin but not GTP hydrolysis by ARF1.

However, a careful electron microscopy analysis of the compartments devoted to SLMV protein sorting in PC12 cells did not reveal any connection between early endosomes-defined compartments and the plasma membrane (de Wit et al., 1999). Moreover, a pathway of internalization directly from the plasma membrane morphologically different from the clathrin-coated pit pathway was not reported in this study. In order to reconcile these somewhat opposite observations it may be speculated that direct SLMV formation from the plasma membrane is mediated by uncoating of a subclass of clathrin-coated vesicles containing exclusively SLMV proteins (de Wit et al., 1999). Thus, two separate pathways of internalization may coexist in PC12 cells, one that

carries both SLMV proteins and non-SLMV membrane proteins, and another that is exclusive for SLMV proteins.

Interestingly, while targeting of P-selectin to SLMVs is inhibited by BFA, implying the requirement for an endosomal intermediate, SLMV targeting of synaptophysin is not affected by the drug (Blagoveshchenskaya et al., 1999b), indicating that synaptophysin may be delivered to SLMVs directly from the plasma membrane, in agreement with what reported by Schmidt et al. (1997). Thus, different SLMV protein may preferentially use one of the two alternative pathways to SLMVs, perhaps depending on the presence of as yet unidentified targeting signals.

1.1.4 Two alternative pathways for SV biogenesis

Incubation of pure SVs under conditions which favour their coating while preventing their uncoating has revealed that ARF1 and the AP3 complex are the only cytoplasmic components required for SV formation from endosomes (Desnos et al., 1995; Faundez et al., 1998). The AP3 coat complex has four subunits with different isoforms, two of which (β3B and γ3B) are neuro-specific (Newman et al., 1995). Only the neuronal form of AP3 can produce SVs from endosomes *in vitro* (Blumstein et al., 2001). These data, in addition to the observation that liver and yeast cytosol could not replace brain cytosol in the reconstitution of vesicle budding from endosomes (Faundez et al., 1998) suggest that SV formation from this compartment may be a function exclusive to neuronal AP3. However, the analysis of the *mocha* mouse, which lacks functional AP3 in all tissues including brain, shows major defects in melanosomes, platelet dense granules and lysosomal traffic, but only subtle neurological phenotypes, such as balance and hearing problems leading to deafness, hyperactivity, seizures susceptibility and abnormalities in theta rhythms (Kantheti et al., 1998 and references within). All these alterations are consistently explained by the missorting of the various cargo proteins that are normally

delivered to intracellular compartments by the AP3 pathway. Despite the observed neurological symptoms, the *mocha* mouse is viable and shows a normal SV population in mossy fiber nerve terminals. This indicates that the AP3 pathway is not essential for SV formation. Instead, other molecules might substitute for AP3 function *in vivo* during SV budding. Alternatively, generation of SVs via the AP3 pathway may represent a minor pathway of SV biogenesis which only operates under certain conditions.

Considerable evidence indicates that SVs can be formed by a pathway that involves AP2, clathrin, and dynamin (Koenig and Ikeda, 1996; Takei et al., 1996; Cremona and De Camilli, 1997; Shupliakov et al., 1997). Elegant electron microscopic studies have shown that the steps mediated by clathrin and dynamin appear to occur at the plasma membrane, although some budding may take place also from internalized membranes (Takei et al., 1996). The α-adaptin-containing AP2 complex is required for the recruitment of dynamin to the endocytotic sites. Consistently, SV recycling is blocked in α-adaptin-deficient Drosophila embryos (Gozalez-Gaitan and Jackle, 1997). The absence of SV retrieval following exocytosis causes the depletion of SVs from the nerve terminals and a corresponding expansion of the presynaptic plasmalemma. The more severe phenotype observed at synapses of the α-adaptin Drosophila mutants compared to the relatively mild neurological phenotype of *mocha* mice, which lack functional AP3 adaptors, implies that the AP2/clathrin-mediated SV formation is more important than that mediated by ARF/AP3.

Therefore, it appears that neurons use two models of vesicle formation, one that generates SVs from the plasma membrane using clathrin and dynamin, and a second that uses AP3 and ARF1 to generate SVs from endosomes.

Neurotransmitter release along developing axons is sensitive to inhibition of ARF activity by BFA, whereas quantal release from mature nerve terminals is BFA-insensitive (Zakharenko et al., 1999). Thus, it seems plausible to hypothesize that

passage of SV membrane components through an endosomal compartment en route to SVs predominates at either early stage of differentiation or specific sites of the neuronal cells, while direct retrieval of SV components from the plasma membrane by the AP2/clathrin pathway operates during SV recycling at the mature synapse, with only a minor contribution of the AP3/ARF pathway (Murthy and Stevens, 1998). A possibility is that SV proteins that escape the normal, non-endosomal route of recycling are internalized into axonal endosomes and are then retrieved via the AP3-mediated route. According to this model, most SLMVs in PC12 are recycled by the AP3 pathway because the cells have not differentiated sufficiently to possess an efficient non-endosomal mechanism (discussed in Blumstein et al., 2001).

An apparent loss of vesicular zinc in hippocampal mossy fibers and in other brain regions is observed in SVs of *mocha* mice. This effect is due to the lack of the SV-associated zinc transport protein ZnT-3 in *mocha* SVs (Kantheti et al., 1998). The absence of vesicular zinc might contribute to al least some of the neurological deficits reported in the *mocha* mouse. However, the evidence that the synaptic terminals from this mouse possess an overall normal morphology has suggested that the synaptic phenotype of the *mocha* mutation is due to a deficiency in a subpopulation of SVs that contain the zinc transporter or in the targeting of a discrete set of SV proteins, rather than a generalized defect in SV biogenesis. Further investigations have indeed allow to show that the ZnT3 transporter and synaptophysin are preferentially targeted to distinct brain SV populations due to their differential interaction with the AP3 complex. While the targeting of ZnT3 to SVs occurs via the AP3 pathway, synaptophysin seems to be directly sorted to SVs from the plasma membrane, likely via the AP2/clathrin pathway (Salazar et al., 2004b). These results are complemented by the evidence that both PC12 cells and neurons assemble molecularly heterogeneous SVs (i.e., high ZnT3 and low synaptophysin levels), supporting the intriguing possibility that distinct SV populations

are generated in a differential manner from the plasma membrane and the endosome. Moreover, heterogeneity in the distribution of vesicular zinc content at the single-neuron level suggests that ZnT3 could be subjected to the control of local mechanisms affecting its transport activity. In fact, the AP3-dependent sorting of either chloride channels or the vesicular glutamate transporter 1 and ZnT3 are reciprocally regulated (Salazar et al, 2004a and 2005). These findings indicate that the biogenesis of SVs is likely to be much more elaborate than previously envisioned, arguing for a sequential process in which multiple, differentially regulated sorting pathway converge on the same vesicle to define its membrane composition and hence its lumenal content.

Supporting evidences for the existence of more than one population of SVs come from developmental studies. SV recycling appears to be tetanus toxin-sensitive and BFA-insensitive at synapses while it is insensitive to tetanus toxin but inhibited by BFA along the developing axon prior to synapse formation (Verderio et al., 1999; Zakharenko et al., 1999). Thus, a change in both vesicle composition and the pathway for SV biogenesis occurs during neuronal development. Based on the preferential targeting of some SV proteins via either the endosomal/AP3 pathway or the plasma membrane/AP2 pathway (Salazar et al., 2004b), it is tempting to propose the existence of a causal relationship between the predominance of a particular mode of SV biogenesis at specific neuronal sites or certain developmental stages and the production of vesicles with distinct composition and function.

1.1.5 Control of SV membrane composition by interaction with adaptor complexes

Some mechanisms must exist to ensure that all nascent vesicles contain the complement of proteins needed for exo-endocytosis. Because the vesicle pool in a nerve terminal can completely recycle in the order of a minute, in the absence of a quality control system

even a low error frequency would rapidly generate a pool of inert SVs. One element of this quality control mechanism is represented by the interaction between VAMP2, which is part of the minimal fusion machinery, and the AP3 complex. Removal of VAMP2 from the endosomal donor membranes prevents the recruitment of the AP3 complex and thus SV formation in a cell-free assay (Salem et al., 1998).

A role for synaptotagmin in quality control during SV formation from the plasma membrane has been suggested by its ability to bind AP2 and direct the nucleation of clathrin-coated pits (Zhang et al., 1994; Haucke and De Camilli, 1999). Synaptotagmin displays a high affinity, calcium-independent interaction of one of its calcium binding domains, C2B, with a region of the μ chain of the AP2 complex (Zhang et al., 1994; Haucke et al., 2000). This binding property appears to be highly conserved in the synaptotagmin family (Li et al., 1995a) and to be dependent on multimerization of the AP2 binding site (Grass et al., 2004). The interaction among cargo proteins, AP2, and synatotagmin appears to be cooperative, since the presence of a peptide containing a tyrosine-based internalization signal enhanced AP2 binding to synaptotagmin *in vitro* (Haucke and De Camilli, 1999). Accurate mutagenesis studies revealed that the AP2 binding site is not needed for synaptotagmin internalization. A latent internalization signal located within the carboxy-terminal of synaptotagmin, which appears to be normally concealed by the C2B domain, is unmasked via a neuro-specific mechanism (Jarousse and Kelly, 2001). The cell-type specificity of this mechanism is demonstrated by prominent surface localization of exogenous synaptotagmin expressed in fibroblasts (Feany et al., 1993). It has been proposed that in neurons and neuroendocrine cells the C2B domain acts as a sensor of intracellular changes in calcium levels following vesicle exocytosis and couples these changes to endocytosis by relieving the inhibition on the internalization signal (discussed in Jarousse and Kelly, 2001). Several AP2 binding proteins such as AP180, endophilin, syndapin, dynamin and intersectin might mediate

the release of this inhibition upon calcium binding to the C2B domain. Two immediate corollaries to this model are the tight coupling between SV exocytosis and re-formation by endocytosis and the generation of SV containing a crucial component of the fusion machinery such a synaptotagmin.

The observation that SVs are depleted at synaptic terminals of synaptotagmin mutants of C. elegans in the absence of transport defects first implicated this protein in SV re-formation after exocytosis (Jorgensen et al., 1995). More recently, a series of elegant experiments based on the live imaging of the pH-sensitive fluorescent chimera synapto-pHluorin provided a quantitative analysis of the kinetics of SV endocytosis in both synaptotagmin I-null Drosophila mutants (Poskanzer et al., 2003) and cultured hippocampal neurons from synaptotagmin I knock-out mice (Nicholson-Tomishima and Ryan, 2004). These approaches allowed to dissect the exo-endocytic process into separate components during synaptic activity. In particular, using fluorescein-assisted light inactivation after normal, synaptotagmin I-mediated, exocytosis, Poskanzer et al. (2003) were able to inactivate synaptotagmin I only during the endocytotic process. The picture emerging from these studies shows that, in addition to regulating exocytosis, synaptotagmin I plays an essential role during compensatory endocytosis at the nerve terminal, thus participating in the maintenance of the SV pool. As discussed above, this model provides a means to link the extent of SV exocytosis and endocytosis.

A possible regulator of AP2 function at synapses is stonin 2, the human homologue of Drosophila stoned B, a presynaptic protein implicated in SV recycling (Martina et al., 2001). Through the binding to synaptotagmin, stonin 2 is recruited to sites of SV recycling where it regulates the AP2-synaptotagmin I interaction during SV endocytosis (Fergestad and Broadie, 2001; Walther et al., 2004). Because of its ability to function as an AP2 dependent sorting adaptor for SV recycling and to interact with a variety of endocytotic proteins, stonin 2 is thought to participate in the general endocytic

machinery that controls SV re-formation from the plasma membrane via the clathrin/AP2 pathway at synapses. In particular, *in vitro* studies (Walther et al., 2004) suggest that the association with stonin 2 renders AP2 incompetent to sort tyrosine motif-containing cargo proteins. This finding led to the proposal that stonin 2, by preventing the constitutive internalization of tyrosine motif-containing plasmalemmal receptors by AP2, may contribute to the specific sorting of proteins from the presynaptic plasma membrane to SVs.

1.2 SYNAPTIC VESICLE RECYCLING AT NERVE TERMINALS

1.2.1 Recycling of SVs occurs by endocytosis

The existence of a recycling pathway that operates in the nerve terminal was inferred by the evidence that SVs become labelled when their exocytosis is stimulated in the presence of an extracellular tracer (Ceccarelli et al., 1972 and 1973; Heuser and Reese, 1973).

The endocytotic retrieval of SVs can be considered a form of high specialization of a process common to all cells. Indeed, since the machinery necessary to load vesicles with neurotransmitter is present within the nerve terminal, SVs can undergo several cycles of exo-endocytosis without the need of being retrogradely transported to the soma for refilling or sorting of its molecular components (Valtorta et al., 1990). Immunofluorescence and immunoelectron microscopy studies showed that at the frog neuromuscular junction SV proteins do not accumulate at the plasma membrane even after high frequency stimulations. In contrast, SV are permanently incorporated in the axolemma when exocytosis is triggered in the presence of a block of endocytosis (Valtorta et al., 1988, Torri-Tarelli et al., 1990). A similar result was obtained when the rate of exocytosis largely exceeded the rate of endocytosis, resulting in the depletion of SVs from the nerve terminal and the accumulation of SV proteins in the plasma membrane (Ceccarelli et al., 1972; Torri-Tarelli et al., 1992).

The lack of intermixing between SV and plasma membrane components may be due either to the absence of complete SV fusion, according to the 'kiss-and-run' mode of SV exo-endocytosis (See below), or to the rapidity and selectivity of retrieval (Mitchell and Ryan, 2004). When full fusion of SVs with the plasmalemma occurs, some sophisticated sorting events have to occur at the nerve terminal. Indeed, complete fusion is accompanied by diffusion of SV components into the axolemma, with mixing of

these components between adjacent synapses (Torri-Tarelli et al., 1990 and 1992; Li and Murthy, 2001; Sankaranarayanan and Ryan, 2001).

1.2.2 Two alternative mechanisms for neurotransmitter release

SVs are continuously regenerated in the nerve terminals following exocytosis in order to repopulate a pool of vesicles large enough to sustain prolonged synaptic activity. New vesicles need to be endowed with all the molecular components which make them suited to fulfil the efficient coupling between the arrival of the stimulus and neurotransmitter release. Importantly, the strategies exploited by SVs to undergo exocytosis, and hence to release neurotransmitters, have crucial consequences bearing on the mechanisms underlying their regeneration.

It is now widely accepted that neurotransmitter release can occur through two different mechanisms, 'full fusion' and 'kiss-and-run'.

The hypothesis of the 'full fusion' mode of neurotransmitter release has been put forward in the early 1970s by Heuser and Reese. According to this model, SVs release their content after full fusion with the plasma membrane. This implies that, during neurotransmitter release, the SV collapses into the presynaptic membrane and is then retrieved via the formation of a coated vesicle (Heuser and Reese, 1973) (Figure 1.1A).

Concomitantly, Ceccarelli and colleagues proposed an alternative model of SV exo-endocytosis (Ceccarelli et al., 1973). In this mode, later named 'kiss-and-run' (Fesce et al., 1994), the SV forms a transient pore with the presynaptic plasma membrane through which the neurotransmitter is released and the vesicle recycles quickly by a direct reversal of the exocytic process, without intermixing with the axolemma. Thus, the vesicle maintains its identity throughout the exo-endocytotic cycle (Figure 1.1B).

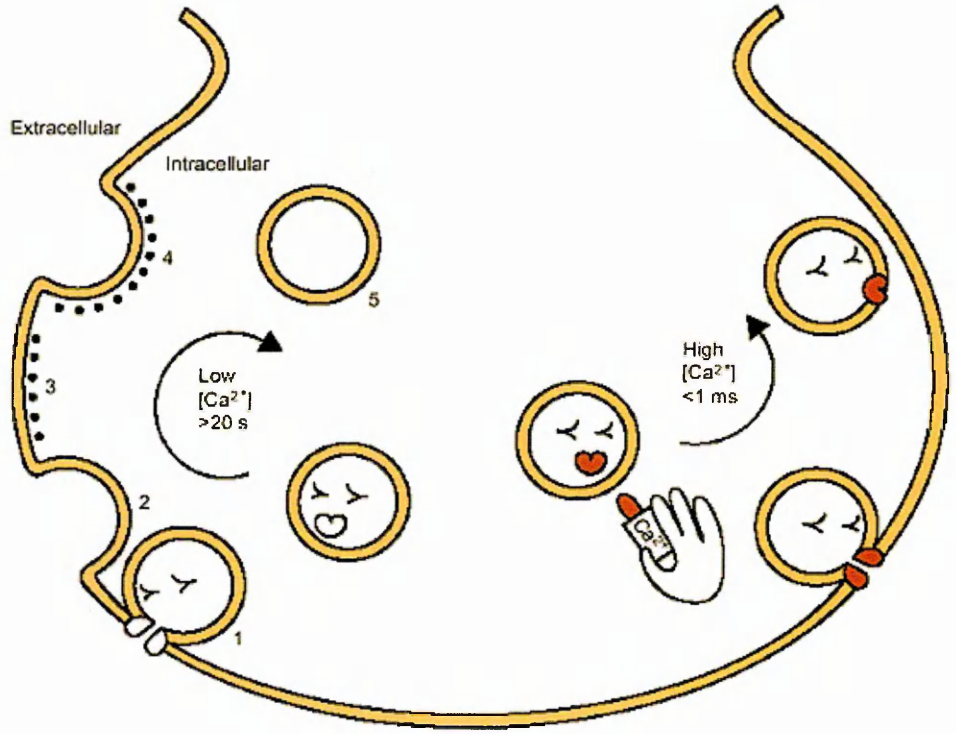

Figure 1.1. Neurotransmitter release can occur by two different mechanisms in the nerve terminal (Fesce and Meldolesi, 1999).
A) After the opening of a fusion pore, the vesicle undergoes complete fusion with the plasma membrane. After neurotransmitter release, the fully fused vesicle is then retrieved by the formation of a coated vesicle. Such mechanism takes more than 20 sec and predominates at low Ca^{2+} concentrations (Heuser and Reese, 1973).
B) The opening of the fusion pore is sufficient for the full discharge of the vesicle content. Therefore, the closure of the pore is sufficient to allow the vesicle to detach from the plasma membrane. This mechanism is faster, since it takes less than 1 msec, and prevails at high Ca^{2+} concentrations (Ceccarelli *et al.*, 1973)

In the past several years a number of experimental evidences which support the (co)existence of the two modes of SV recycling have been reported (for a review see Valtorta et al., 2001).

Measurements of the kinetics of SV endocytosis at synapses formed by hippocampal neurons in culture reveal that at least two different mechanisms of retrieval take place in the nerve terminals: a fast process with a time constant shorter than 1 sec, and a slower process with a time constant of 20-30 sec (Klingauf et al.,1998). A major breakthrough was achieved by Alvarez de Toledo and colleagues (Ales et al., 1999). These Authors

demonstrated that in rat chromaffin cells the complete release of the secretory granule content does not require full fusion. Indeed, the fusion pore reversibly opens up to a size corresponding to a conductance above 1.5 nS, enough to completely discharge the full content of the vesicle, and can still close quickly.

Although the protein machinery which mediates exocytosis of granules in chromaffin cells is similar to that operating in neurons, a significant difference between the two systems is represented by the fact that the local Ca^{2+} concentration near the sites of exocytosis is much higher in synapses than in chromaffin cells (Neher, 1998). At low Ca^{2+} concentrations re-closure of the pore is unlikely and secretory granules preferentially undergo full fusion with the plasma membrane. Remarkably, at increasing Ca^{2+} concentrations the probability and the rate of re-closure of the fusion pore increase, so that kiss-and-run events are favoured.

Interestingly, a marked increase of kiss-and-run following intense stimulations was observed not only after the rise of Ca^{2+} levels (Ales et al., 1999; Pyle at al., 2000), but also when exocytosis was stimulated in Ca^{2+}-independent fashions using either hypertonic solutions (Pyle et al., 2000) or phorbol esters (Cousin and Robinson, 2000). Taken together, these results suggest that, when the demand for vesicle recycling increases, synaptic terminals make greater use of the fast and highly economical kiss-and-run mode.

1.2.3 Clathrin-mediated synaptic vesicle endocytosis

According to the classical model of exo-endocytosis proposed by Heuser and Reese (1973), collapse of the SV into the plasmalemma is followed by the assembly and endocytosis of clathrin-coated vesicles which fuse with an endosomal compartment, from which new SVs will bud off. The main function for clathrin-coated vesicles in the brain is to recapture SVs after exocytosis. Indeed, a near-complete inventory of the

known SV proteins has been detected by tandem MS analysis of brain clathrin-coated vesicles, with a lack of abundant presynaptic plasma membrane proteins (Blondeau et al., 2004).

The formation of clathrin-coated vesicles in the nerve terminals appears to rely on the function of the adaptor protein AP2, which initiates the recruitment of the endocytotic machinery. Direct interaction between AP2 and the cytoplasmic C2B domain of synaptotagmin is likely to guarantee the specificity of the endocytotic pathway for SVs re-formation (See above). In addition to AP2, a brain specific monomeric adaptor protein, AP180, has been identified in association with clathrin-coated vesicles from nerve terminals (Ye et al., 1995). AP180 is thought to act in concert with AP2 to regulate clathrin-mediated endocytosis of SVs (Schmid, 1997). Consistently, Drosophila mutants lacking the AP180 homologue LAP display severe defects in endocytosis, resulting in the depletion of SVs from the nerve terminals (Zhang et al., 1998). The injection of antibodies against AP180 into the giant presynaptic terminals of the squid results in the block of synaptic transmission and in a strong increase in the surface area of the terminal (Morgan et al., 1999). At variance, the C. elegans homologue of AP180, UNC-11, appears dispensable for clathrin-mediated endocytosis but required to maintain the correct size of endocytosed SVs (Nonet et al., 1999).

Assembly of the clathrin matrix is a GTP- and ATP-dependent process, whereas the subsequent invagination of the coated vesicle requires exclusively GTP-dependent activities. Fission of the coated vesicle again relies on both ATP- and GTP-dependent processes. The GTPase activity which mediates the fission step is provided by the atypically large and modular GTPase dynamin, which is required for a variety of endocytotic pathways, including phagocytosis, clathrin/caveolae-mediated endocytosis and some forms of clathrin/caveolae-independent endocytosis (Conner and Schmid, 2003). The Drosophila mutants of the dynamin homologue, shibire, display a

temperature-sensitive block of endocytosis resulting in the rapid depletion of the releasable SV pool (Kuromi and Kidokoro, 1998).

The function of dynamin has been elucidated by ultrastructural analysis of the dynamin rings formed around the neck of invaginating coated pits. These transient structures, are stabilized by GTPγS, a non-hydrolysable analogue of GTP (Takei et al., 1995a). The current model envisioning the action of dynamin in endocytosis postulates that the protein is recruited to coated pits in its GDP-bound state. After GDP/GTP exchange, dynamin assembles at the neck of the coat, forming a helical collar. The hydrolysis of GTP results in a conformational change that precedes fission, followed by the dissociation of dynamin from the complex (Conner and Schmid, 2003). The ability of dynamin to promote tubulation and vesiculation of acidic liposomes suggest that it might act as a molecular spring to pop the forming SV off (Stowell et al., 1999). However, this model of dynamin action has been questioned (Sever at al., 1999). Instead of being a force-generating GTPase, dynamin is proposed to act as a molecular switch that recruits, in a GTP-dependent manner, an essential component of the fission machinery to the neck of the nascent endocytic vesicle. In the case of the SV, this component has been proposed to be the lipid modifying enzyme SH3P4/endophilin 1 (Schmidt et al., 1999; Hannah et al., 1999; Huttner and Schmidt, 2000).

The GTPase activity of dynamin can be modulated by several proteins that could promote or stabilize it. In particular, a proline/arginine rich domain of dynamin may mediate its binding to Src homology 3 (SH3) modules. A neuronal SH3 containing partner of the neuro-specific dynamin I is amphiphysin. Since amphiphysin interacts with both dynamin I and the AP2 complex, it might be critical to couple the formation of coated pits to the fission machinery (De Camilli and Takei, 1996). In keeping with this model, injection of either the SH3 domain of amphiphysin or a dynamin I form

lacking the amphiphysin-interacting domain arrests endocytosis at the stage of deeply invaginated, 'uncollared' clathrin-coated pits (Shupliakov et al., 1997).

In addition to binding to dynamin I, the SH3 domain of amphiphysin can also interact with synaptojanin-1, an inositol-phosphate phosphatase enriched in nerve terminals (McPherson et al., 1996). Moreover, dynamin I itself binds to phosphoinositides, albeit with lower affinity (Schmid et al., 1998). Furthermore, the GTPase activity of dynamin is enhanced by phosphorylation by PKC. This regulatory mechanism is inhibited by the action of both the phosphatase calcineurin and casein kinase II (Robinson, 1994).

As aforementioned, the dynamin interacting protein endophilin 1 might play a pivotal role in the regulation of the fission machinery. Using a perforated PC12 cells system that reconstitutes the formation of SLMVs from the plasma membrane, endophilin 1 was found to be a rate-limiting component of the cytoplasmic machinery mediating the vesicle budding and fission processes (Huttner and Schmidt, 2000). Endophilin 1 was found to possess a lysophosphatidate acyltransferase activity. Recombinant endophilin 1 stimulates SLMV formation by mediating the conversion of lysophosphatidic acid to phosphatidic acid. Importantly, an endophilin 1 mutant lacking the SH3 domain, and hence incompetent for dynamin binding, but still exhibiting enzymatic activity, is unable to promote SLMV formation (Schmidt et al., 1999). The mechanism by which the recruitment of endophilin 1 to the neck of the nascent SV stimulates the fission event remains to be established. It has been proposed that the conversion of lysophosphatidic acid, an inverted cone-shaped lipid, to phosphatidic acid a cone-shaped lipid, might promote fission by affecting the curvature of the cytoplasmic membrane leaflet (Huttner and Schmidt, 2000).

1.2.4 Pathways of SV recycling at synapse

Altogether, the data reported above suggest that a highly entangled circuit of regulation of SV endocytosis operates within the nerve terminal. An extended high-frequency stimulation (15 min at 10 Hz) at the frog neuromuscular junction leads to the appearance of HRP-filled cisternae/vacuole-like structures which were interpreted as the organelles from which SVs form during an extended (1 h) period of recovery (Heuser and Reese, 1973). On the contrary, nerve terminals stimulated extensively at lower frequencies (4 h at 2 Hz) reveal mainly HRP-filled vesicles even in the absence of a post-stimulation recovery period (Ceccarelli et al., 1973). Nevertheless, vacuolar structures were present in these terminals, although they were not engaged in SV recycling under the stimulation protocol applied.

Two different models of clathrin-dependent endocytosis have been proposed (Figure 1.2):

1- The 'classical model' postulates that the coated vesicle undergoes ATP-dependent loss of the clathrin lattice and fuses with the endosomal compartment, from which new vesicles are formed through an independent sorting process (Heuser and Reese, 1973);

2- The alternative model proposes that SVs can form directly from clathrin-coated vesicles by loss of the coat, thereby reducing the number of steps needed for the endocytotic process (De Camilli and Takei, 1996). According to this model, endosome-like intermediates in nerve terminals may originate from deep invaginations of the plasmalemma and are not preexisting internal structures that act as acceptor membrane of endocytotic vesicles. These invaginations might either maintain a narrow connection to the plasmalemma or be eventually internalized. Whatever the case, these vacuoles are likely to be molecularly very similar to the plasmalemma rather than to endosomes. Thus, SVs are directly

produced in a single clathrin coat-mediated budding and dynamin-mediated fission step from either the presynaptic plasma membrane, or deep plasma membrane invaginations, or both. This would explain the similarity between the molecular composition of clathrin-coats observed on the vacuole membranes and at the nerve terminal surface (Takei et al., 1996; Gad et al., 1998).

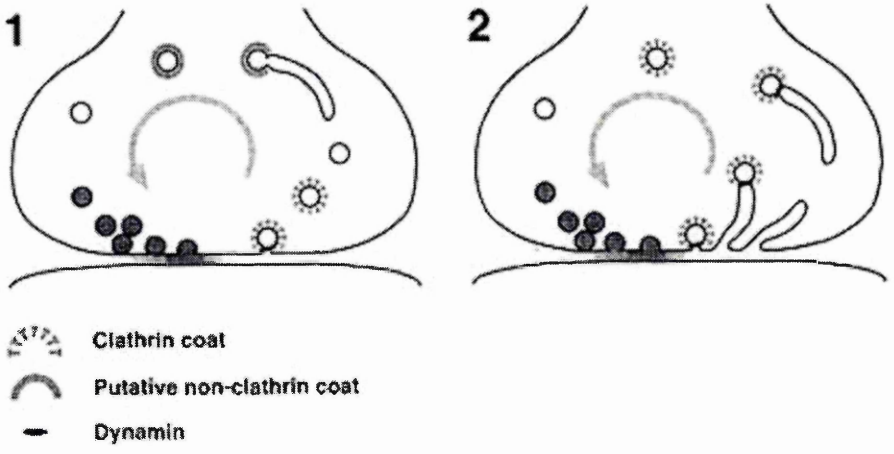

Figure 1.2. Alternative models for clathrin-dependent SV endocytosis (De Camilli and Takei, 1996).
Two models have been proposed for clathrin-dependent recycling of the SVs at the nerve terminal. 1) The endocytic coated vesicle undergoes an ATP-dependent loss of the clathrin lattice and fuses with an endosomal compartment, from which new SVs are formed through an independent sorting process.
2) Direct generation of new SVs from either plasma membrane invaginations or endosome-like intermediates. SVs derive from coated vesicles by simple loss of the clathrin coat.

It is immediately apparent that the modification of the Heuser-Reese model proposed by De Camilli and colleagues brings the 'classical model' and the 'kiss-and run' model closer, yet preserving a central role for clathrin and the participation of vacuolar structures during intense stimulations. Moreover, it provides a conceptual framework to reconcile data indicating that both bulk, non-selective endocytosis mediated by large

vacuoles (Koenig and Ikeda, 1989) and selective clathrin-mediated endocytosis (Heuser and Reese, 1973) participate in SV recycling.

The idea that two separate, differentially regulated mechanisms for SV re-formation via endocytosis coexist at the nerve terminal, one for direct SV recycling from the plasma membrane and one for recycling via endosomal intermediates, is supported by the ultrastructural analysis of the Drospohila mutant *shibire*, that harbours a temperature-sensitive mutation of the dynamin gene (Koenig and Ikeda, 1996). This hypothesis implies the existence, at least in the flies, of two recycling pathways with different ion sensitivity. One pathway emanates from the active zone of exocytosis, displays fast kinetics and is sensitive to high Mg^{2+}/low Ca^{2+} saline. It involves small clusters of vesicles that are observed at the active zones. The formation of these vesicles does not include intermediate structures, such as coated pits, coated vesicles, or cisternae, and might be accomplished by a direct pinch-off at the plasma membrane. At variance, the second pathway emanates from sites away from the active zones, has a slower kinetics, is unaffected by the high Mg^{2+}/low Ca^{2+} saline and involves coated collared pits. The localization of the machinery for clathrin-mediated endocytosis, including α-adaptin and dynamin, to areas of the presynaptic plasma membrane that are distinct from the regions that are predicted to be active zones of exocytosis suggests that the AP2-mediated SV retrieval operates preferentially in the pathway of endocytotic SV re-formation, involving coated vesicles outside the active zones (Gonzalez-Gaitan and Jackle, 1997; Roos and Kelly, 1999). This would implicate that SVs are recycled via the kiss-and-rune mode (fission only) at the active zone, while a clathrin plus dynamin-mediated type of retrieval (budding plus fission) operates at the nonactive zones, in keeping with the 'classic model' of SV recycling.

However, the actual situation is likely to be more complicated, and the different pathways of SV recycling might be preferentially used at certain synapses but absent in

others. At snake motor terminals all of the endocytotic SVs observed after a brief, low-frequency stimulation applied at reduced temperature are clathrin-coated and clustered near active zones, arguing against the occurrence of the kiss-and-run mode in this preparation (Teng and Wilkinson, 2000). The unusually rapid SV retrieval (time constant ~1 sec) observed at ribbon synapses of retinal bipolar neurons, relies on an unconventional mechanism of activity-dependent endocytosis leading to the formation of transient, large, plasmalemma-derived vacuoles, which are likely to subsequently bud off SVs (Paillart et al., 2003). An analogous mode of bulk endocytosis, resulting in the appearance of endosomal-like structures which then give rise to new SVs, was reported in other systems. However, this mechanism is not likely to make a substantial contribution to recycling during low-frequency stimulation (Leenders et al., 2002; de Lange et al., 2003) (Figure 1.3).

The function of endosomal-like elements is debated. The activity-dependent bulk membrane uptake observed at the giant terminals of retinal bipolar neurons was shown to share common features with macropinocytosis described in non-neuronal cells, and has been linked to the regulation of the structural plasticity of the nerve terminal rather than to the maintenance of SV cycle during prolonged stimulations (Holt et al., 2003). Moreover, it is not clear whether the internal vacuoles implicated in the clathrin-mediated pathway are distinct from endosomes. In the absence of a detailed molecular characterization of these membrane elements (see Marxen et al., 1999) the question remains unanswered. If the vacuolar structures described in several studies do not display an endosomal identity, endosomes, which play a major role in SLMV formation in neuroendocrine cells, are dispensable for SV recycling at synapse. Thus, although the presence of endosomes in the nerve endings is documented by several studies, their function might be limited to the *de novo* assembly of newly synthesized proteins into a mature SV.

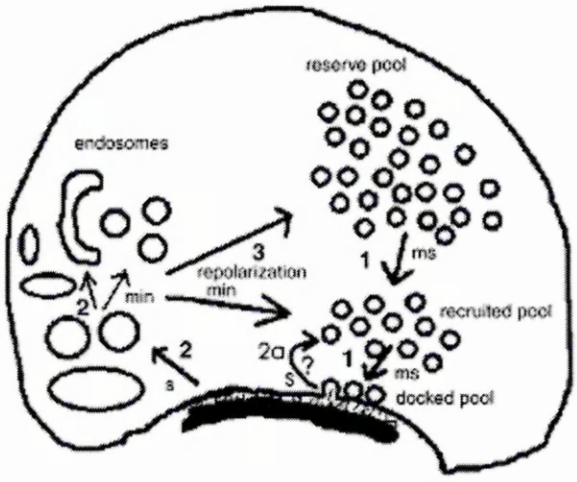

Figure 1.3. Model of depolarization-induced SV recycling. (Leenders et al., 2002) Short (ms) depolarization activates the transfer of SVs from the reserve to the recruited pool, and SV docking at the active–zones (1). Retrieval of vesicle membrane after prolonged depolarization (s) occurs either directly as SVs (2a), or as larger endosome-like organelles (2) which eventually increase in number (min). Already during, and mostly after, depolarization, SVs are generated from endosomal-like compartments (3).

1.2.5 Do specialized endosomes operate in SV recycling/biogenesis?

It was hypothesized that *de novo* formation of SVs may occur from a specialized class of endosomes (Kelly et al., 1993). In spite of these expectations, the morphological characterization of the endocytic pathway in PC12 cells revealed that endosomal compartments in this cell line are very similar to those of non-neuroendocrine cells (de Wit et al., 1999). Neither the morphology of the distinct endocytic intermediates nor the comparison between the distribution patterns of SLMV and non-SLMV proteins provide any clues for the existence of a specialized endosome dedicated to SLMV biogenesis/recycling. In agreement with earlier studies (Cameron et al., 1991; Linstedt and Kelly, 1991b; Grote et al., 1995; Grote and Kelly, 1996) synaptophysin and VAMP2 were mainly found on early endosomes as opposed to late endosomes and lysosomes, and especially on the tubular extensions of early endosomes, which are

identified as the likeliest candidates for the intracellular SLMV donor compartment (de Wit et al., 1999).

In neurons, electron microscopy studies have shown the presence of extensive networks of tubular endosomes in dendrites and cell bodies whereas, in axon, early endosomes were found exclusively in the presynaptic terminals and in varicosities (Parton et al., 1992). Consistent with these observations, the axon shaft of mature neurons is largely devoid of endocytic activity and internalization only occurs at the nerve terminals. In contrast, the entire dendritic plasma membrane of mature neurons shows high endocytic activity. Thus, the axonal and somatodendritic domains of polarized hippocampal neurons seem to possess distinct endocytic circuits (Parton et al., 1992), although they share common components of the endocytic machinery, such as the small GTPase Rab5 (de Hoop et al., 1994) and the SNARE protein syntaxin 13 (Prekeris et al., 1999). Endosomes found in the axons and nerve terminals in primary hippocampal neurons (Mundigl et al., 1993) and in the neurites in differentiated PC12 cells (Bonzelius et al., 1994) notably lack the transferrin receptor, which is associated with the soma and dendrites.

BFA, which inhibits ARF protein function, has been useful in characterizing functionally specialized endosomes in polarized cells. BFA induces a massive tubulation of transferrin receptor-containing endosomes in the somatodentritic region, whereas no obvious morphological changes are produced in axons (Mundigl et al., 1993). Moreover, in cultured hippocampal neurons the transcytotic transfer from dendritic endosomes to the axons is sensitive to BFA (de Hoop et al., 1995). Differences in the sensitivity to BFA action indicates that the molecular composition of the two classes of endosomes may be distinct.

So far, the only clear molecular difference between the endosomal compartments found in the axonal and somatodendritic domains of neurons is the exclusive association of

EEA1 with Rab5-positive endosomes of the somatodendritic domain of polarized hippocampal neurons (Wilson et al., 2000). EEA1 is one of the best characterized early endosome marker and vital effector of Rab5 (Christoforidis et al., 1999). In the light of the more widespread distribution of Rab5 relative to EEA1, the nature of the effector associated with EEA1-negative presynaptic endosomes remains to be investigated.

While the role of early endosomes in the control of SV recycling is well supported, the function of tubulovesicular recycling endosomes containing sytaxin 13 that are found associated with the axonal domain of polarized hippocampal neurons is less clear (Prekeris et al., 1999). These endosomes are excluded from synaptic contacts and do not colocalize with SV antigens both in mature and immature neurons, suggesting that they are distinct from the axonal endosomes positive for Rab5. In the light of the role of syntaxin 13 in non-neuronal cells, where it controls transferrin receptor recycling (Prekeris et al., 1998), it is tempting to speculate that syntaxin13 is the SNARE involved in homotypic endosome fusion.

Together, these results have led to proposal that the somatodendritic endosomes (transferrin receptor-and EEA1-positive, BFA-sensitive) play a 'housekeeping' role, whereas the presynaptic endosomes (transferrin receptor- and EEA1-negative, BFA-insensitive) play a unique role in generating SVs (Parton and Dotti, 1993; de Hoop et al., 1994). However, this picture contrasts with the reported BFA-sensitivity of SLMV biogenesis in PC12 cells (Faundez et al., 1997) and SV recycling in developing axons of frog motoneurons and immature mammalian neuromuscular junctions (Zakharenko et al., 1999; Polo-Parada et al., 2002). This discrepancy is only partially explained by assuming that the BFA-sensitive pathway implicated in SV biogenesis in PC12 and immature neurons represents a more elementary form of process that is replaced during nerve terminal maturation with a more efficient and unique neuronal process (See Zakharenko et al., 1999). Indeed, at variance with the studies mentioned above, BFA

does not appear to have any effect on recycling of SVs in immature hippocampal neurons, as demonstrated by uptake of an antibody against synaptotagmin (Matteoli et al., 1992; Mudigl et al., 1993), possibly reflecting a different timing in the maturation of the neurosecretory apparatus in peripheral and central synapses.

Although the precise contribution of axonal endosomes to SV formation remains to be established, the two small GTPases Rab5 and Rab4, which in non neuronal cells regulate transport to and through early endosome, respectively (Bucci et al., 1992; van der Sluijs et al., 1992), appear to govern SV recycling/formation.

Rab4 is associated with the early endosomal precursors of SLMVs in PC12 cells, and regulates budding of SLMVs from these compartments. In addition, Rab4 regulates the exit of constitutive recycling proteins from early endosomes in PC12 cells. However, Rab4 does not appear to affect the sorting of VAMP2 from transferrin receptor, suggesting that it acts distally to the primary sorting process of the two transmembrane proteins (de Wit et al., 2001).

The existence of a Rab5-dependent pathway involved in the trafficking of several SV proteins to the nerve terminal has been confirmed by the expression of Rab5 mutants in cultured hippocampal neurons. The inhibition of Rab5 activity severely impaired the axonal transport of SV proteins (Kanaani et al., 2004). Axonal Rab5-positive endosomes have been also implicated in the differential sorting of the amyloid precursor protein (APP) and SV components, which are internalized together via the same clathrin-coated vesicle at the nerve terminals and then directed to either the retrograde transport route or the recycling SV pool, respectively (Marquez-Sterling et al., 1997).

Rab5 was found on synaptophysin-containing vesicles immunoisolated from SV preparations (de Hoop et al., 1994; Fischer von Mollard, 1994). Together with the description of Rab5-positive compartments (de Hoop et al., 1994) in both axon and

dendrites of cultured hippocampal neurons, these results were the first to suggest that SV components recycle via early endosomes and argued for the view that early endosomes participate in the biogenesis of SVs. Monitoring of the endosomal compartments in Drosophila neuromuscular synapses revealed that the Rab5-positive endosomes are required to sustain SV exo-endocytosis during intense synaptic activity. Altering Rab5 function influences the synaptic efficacy. Importantly, these effects are not due to a change in the readily releasable pool size, but rather to a change in the release probability of SVs (Wucherpfennig et al., 2003). An attractive explanation as to how the membrane exchange between vesicles and the endosome could affect the release probability of SVs is to hypothesize that endosome function is required to control the protein and lipid composition of SVs, either by adding to and removing from budding vesicles newly synthesized and aged components, respectively, or allowing the differential sorting of SV components in order to modify the molecular make up of the vesicles. At synapses, Rab5 has also been implicated in the maintenance of the uniform size of SVs by prevention of homotypic vesicle fusion (Shimizu et al., 2003).

1.3 SORTING AND ASSEMBLY OF SYNAPTIC VESICLE COMPONENTS

SVs purified from the brain have a distinct and fairly simple protein composition (Huttner at al., 1983). The observation that SVs contain a very restricted set of membrane proteins implies the existence of a sorting process. The sorting of SV proteins is defined as their segregation from non-SV proteins during either the formation of a vesicle from a donor membrane or the formation of distinct microdomains in the plane of the donor membrane (Hannah et al., 1999).

In analogy to the raft concept (Simons and Ikonen, 1997), the enrichment of specific lipids within membrane microdomains might allow, by means of distinct protein-protein and protein-lipid interactions, to segregate SV components from resident proteins of the donor membranes. If all the SV membrane constituents spontaneously pre-assemble in the specialized microdomain with the correct stoichiometry, the formation of the SV itself could theoretically proceed as a default single budding step from the highly pre-sorted donor membrane (Figure 1.4, upper panel).

Alternatively, the formation of mature SVs, enriched in all the components needed for efficient neurotransmitter release, might rely on a stepwise process requiring multiple cycles of SV exo-endocytosis undertaken either along the axon or at the presynaptic terminals. Indeed, extrapolating from the study of SLMV biogenesis (Regnier-Vigouroux et al., 1991), several passages through the plasma membrane and endosomal compartments might precede the incorporation of SV proteins into the mature vesicles (Figure 1.4, lower panel). The possibility that removal of vesicle components, rather than insertion of new proteins, drives the maturation of the organelles is suggested by the study of granules involved in peptide hormone secretion in a pituitary cell line (Eaton et al., 2000). An ARF-mediated sorting pathway is responsible for functional

remodelling of the granule membrane. Several trafficking proteins, including VAMP4 and synaptotagmin IV, are sorted away during the transition from immature granules, which exhibit unregulated secretion after budding from the TGN, to mature, regulated exocytotic carriers. The removal of synaptotagmin IV, which inhibits calcium-triggered exocytosis (Littleton et al., 1999) provides a crucial switch between unregulated and regulated secretion during granule maturation.

Although a direct evidence is lacking, the two general mechanisms of protein sorting to SVs described above are not mutually exclusive. Rather, they are likely to work in concert during the SV life cycle, both at the plasma membrane and in endosomal compartments.

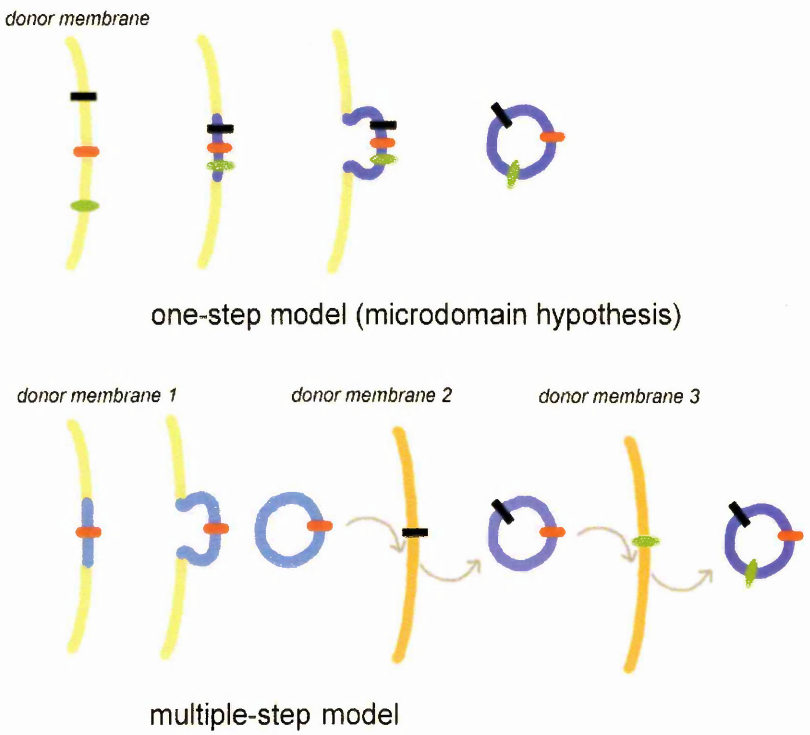

Figure 1.4. Models for the assembly of SV components.
(Upper panel, one-step model) The lateral organization of microdomains with distinct lipid composition allows SV components to be enriched in these specialized membrane elements and eventually sorted into the same vesicle in a single budding step from the donor membrane. (Lower panel, multiple-step model) Multiple cycles of exo-endocytosis through various donor compartments, either the TGN, or endosomes, or the plasma membrane, are needed in order for the maturing SV to be endowed with the complete complement of membrane proteins.

1.3.1 Sorting signals

Signal sequences are thought to mediate the sorting of many proteins to cellular compartments. At present, two major groups of sorting signals have been identified. The first group comprises tyrosine-based signals, which usually conform to the consensus sequences YXXØ (where X is any amino acid, and Ø is a strong hydrophobic amino acid) or FXNPXY. The second group of sorting signals contains di-leucine/di-hydrophobic signals, in which one of the leucines can be substituted by isoleucine, methionine, or valine (Bonofacino and Traub, 2003).

Several studies have identified domains and motifs necessary for the correct localization of SV proteins. However, no common motif that could serve as a universal targeting signal has been described. The absence of a common sorting signal element in SV proteins contrasts with the targeting to other organelles, where many distinct proteins use the same sorting signals. At least two different mechanisms of SV protein sorting could account for the lack of a universal SV targeting signal. First, distinct components of SVs may be sorted independently by distinct processes. In keeping with this possibility, al least some SV proteins seem to be transported down the axon in different carriers (Okada et al., 1995) and mutations affecting the sorting of a SV protein leave the other components unaffected (Nonet et al., 1999). Second, the sorting information might be present in a few proteins, with other components being secondarily targeted to SVs by interaction with these proteins (Bennett et al., 1992; See also Martin, 1999). More likely, a combination of these two mechanisms is used to target SV proteins.

Several lines of evidence indicate that different proteins use different pathways to be targeted to the SV. This suggests that each SV protein contains multiple signals that control its trafficking along the various pathways. These signals may mediate different sorting steps at the TGN, plasma membrane or endosomes. The situation is further complicated by the partial overlap between signals responsible for the polarized traffic

of SV proteins and signals responsible for their targeting to SVs. In keeping with these ideas, multiple signal sequences have been found in various SV proteins. Here, I provide an overview of the sorting determinants so far identified in some of the SV proteins.

In the past ten years, a number of different laboratories have contributed to a comprehensive study of the sorting determinants involved in the targeting of VAMP2 to SVs. VAMP2 does not possess a signal sequence to be directed to a translocation apparatus of the ER membrane (Kutay et al., 1993). Insertion of VAMP2 into the ER membranes occurs postranslationally and is mediated by the interaction of a conserved bipartite carboxy-terminal motif with an as yet unidentified ER protein(s) (Kutay et al. 1995; Kim et al., 1999). In PC12 cells, after insertion into the ER membrane,VAMP2 is transported via the Golgi apparatus to SLMVs (Kutay et al., 1995).

A GFP-tagged chimera of VAMP2 has been exploited to visualize the post-Golgi traffic of the protein in polarized hippocampal neurons in culture (Sampo et al., 2003). Exogenous VAMP2 is delivered equally to the surface of both axons and dendrites, but is preferentially endocytosed from the dendritic plasma membrane. Thus, selective endocytosis rather than selective delivery appears to be responsible for the axonal polarization of VAMP2. These results have been corroborated by the use of a point mutant of VAMP2 which remains blocked at the plasma membrane in association with t-SNARE proteins due to the inhibition of SNARE complex dissociation (Martinez-Arca et al., 2004). Expression of such mutant showed that after leaving the Golgi VAMP2 is directly targeted to the plasma membrane of both axons and dendrites.

These observations are in keeping with the results obtained in C. elegans by genetic ablation of UNC-11, the homologue of the mammalian neuro-specific clathrin-adaptor protein AP180. This study uncovers a role for endocytosis from the plasma membrane in the sorting of VAMP2 to SVs. In the *unc-11* mutants, VAMP2 is mislocalized to the

neuronal plasmalemma, no longer being exclusively present on SVs. This phenotype appears to be specific for VAMP2, since is not observed for other SV proteins, including synaptogyrin and synaptotagmin, which remain localized to SVs (Nonet et al., 1999). Therefore, either a direct or indirect interaction of UNC-11 with VAMP2 is responsible for the recruitment of VAMP2 to nascent SVs. Whether the targeting of VAMP2 to SVs occurs in a single step via UNC-11-mediated endocytosis from the plasma membrane or whether it requires a passage through endosomal compartments (See Salem et al., 1998) is unclear.

The trafficking route responsible for VAMP2 targeting to SVs has been further dissected by expressing in hippocampal neurons a chimera consisting of the cytoplasmic domain of VAMP2 (aa 1-93) fused to the amino-terminus of the complete transferrin receptor (West et al., 1997). In striking contrast to the normal distribution of the transferrin receptor, which is localized in the somatodendritic region and excluded from the axon, the chimera is directed to the axon, where it primarily accumulates at presynaptic sites, consistently with the presence of a synapse-targeting signal in the cytoplasmic domain of VAMP2. However, in nerve terminals the VAMP2-transferrin receptor chimera is associated with endosomal compartments rather than SVs. A further sorting decision, mediated by a separate, inhibitory signal within the cytoplasmic domain of VAMP2 (aa 61-70) is required in order for VAMP2 to be targeted from axonal endosomes to SVs. Consistently, deletion of this sequence results in shifting of the chimera to SVs. The same inhibitory signal had been identified by Grote et al. (Grote at al., 1995; Grote and Kelly, 1996) during a detailed analysis of SLMV targeting and endocytosis efficiencies exhibited by a panel of deletion and point mutants of an epitope-tagged VAMP2 (VAMP2-TAg) expressed in PC12 cells. These studies allowed the identification of a sequence (aa 41-50) within the predicted α-helix1 of the cytoplasmic domain responsible for VAMP2 targeting to SLMVs. A point mutation

(M46A) within this region severely impairs SLMV targeting. In contrast, amino acid substitutions which increase the hydrophobicity of the helix (e.g., N49A) enhance the targeting (Grote et al., 1995). Remarkably, while the positive signals (aa 50-41, Met46) required for VAMP2 targeting to SLMV are also indispensable for endocytosis, the negative signals (aa 61-70, Asn49, Asp44) are dispensable, since their mutations, which enhance targeting to SLMVs, do not affect the endocytosis rate. Importantly, none of these elements conforms to previously identified endocytosis signals (Grote and Kelly, 1996). These results provide evidence that endocytosed VAMP2 is not directly sorted to SLMVs from the plasma membrane, but is internalized primarily to intracellular compartments en route to SLMVs.

Altogether these studies are consistent with the view that VAMP2 undertakes a tortuous trafficking route, regulated by multiple SV targeting and SV exclusion signals within its primary sequence, in order to reach its final destination (Figure 1.5). It remains to be established whether a precise pattern of protein-protein interactions mediates the activation or the concealing of the various targeting signals of VAMP2 at different stages during its intracellular transport.

Extrapolation to neurons of the results obtained in PC12 cells as to the role of targeting signals should however be considered cautiously. Indeed, significant differences have been reported between PC12 cells and neurons from intact animals. Expression of targeting mutants of VAMP2 identified in PC12 cells (M46A and N49A) rescued the lethal phenotype of C. elegans synaptobrevin mutants, and the exogenous proteins localized indistinguishably from the wild type VAMP2, implying that the identified sequences do not play critical roles in VAMP2 sorting *in vivo* (Zhao and Nonet, 2001).

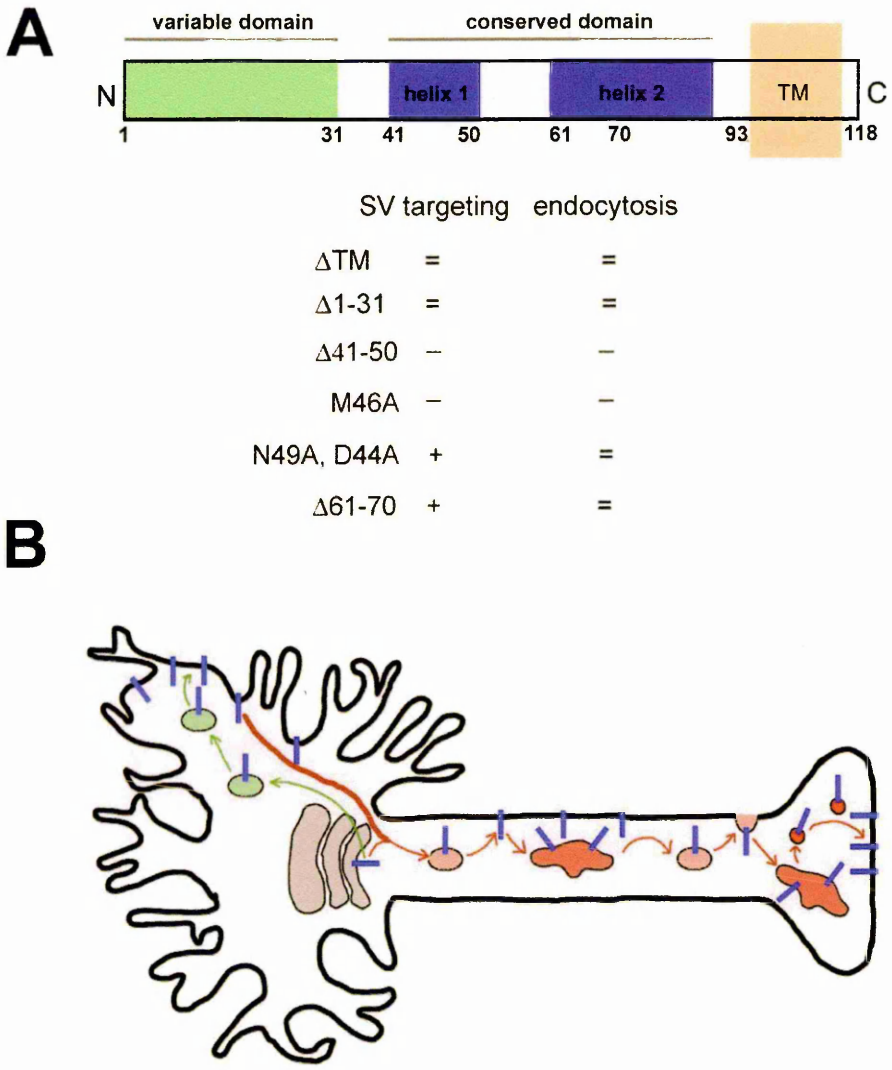

Figure 1.5. Sorting motifs and trafficking routes of VAMP2.
A) Schematic representation of VAMP2 (TM, transmembrane domain). The effects of the various mutations on either VAMP2 targeting to SVs or endocytosis are indicated.
B) After exiting the TGN, VAMP2 (blue bar) is delivered to the surface of both axons (red line) and dendrites (green line), but is readily endocytosed from the dendritic plasma membrane and possibly re-directed to the axon. Once delivered to the presynaptic terminals VAMP2 cycles constitutively between the plasma membrane and endosomes. Concealing of a negative signal occurs at the level of the endosomal compartment to remove VAMP2 from this circuit and target it into the newly assembled SV.

Targeting of synaptotagmin I to presynaptic sites is mediated by palmitoylation of amino-terminal cysteines present in the junction between the transmembrane and cytoplasmic regions of the protein (Kang et al., 2004). Interestingly, the palmitoylated amino-terminus of synaptotagmin VII was not sufficient for targeting to presynaptic

sites, implying that specific motif may be present only in a subset of members of the synaptotagmin family. Presynaptic localization of palmitoylated synaptotagmin I is correlated with its internalization efficiency, suggesting the involvement of an endocytotic pathway in synaptotagmin targeting to SVs.

Synaptotagmin I internalization relies at least on two distinct signals. A strong, unconventional, internalization motif is present in the carboxy-terminal domain of the protein and appears to be regulated by the C2B domain (Jarousse and Kelly, 2001; See above). In addition, the neuro-specific intralumenal N-glycosilation of synaptotagmin I acts in concert with the cytoplasmic domain to direct the protein from the plasma membrane to SVs (Han et al., 2004). Consistently, synaptotagmin VII, which is not N-glycosilated, is targeted to plasma membrane.

The pathway followed by synaptotagmin I to be delivered to SVs also includes the passage through an endosomal compartment. The carboxy-terminal domain of synaptotagmin I contains a di-hydrophobic motif (ML) which, although dispensable for synaptotagmin I internalization, yet supports part (20%) of its trafficking to SLMVs in PC12 cells. All the traffic of synaptotagmin I to SLMVs mediated by the ML motif is BFA sensitive, strongly suggesting the involvement of an AP3-dependent route through an endosomal compartment (Blagoveshchenskaya et al, 1999a).

Similarly to synaptotagmin I and VAMP2, the targeting of GAD65, the smaller isoform of the GABA-synthesizing enzyme glutamate decarboxylase, to SV presynaptic clusters is controlled by multiple signals (Kanaani et al., 2002). GAD65 is synthesized as a soluble hydrophilic molecule but undergoes amino-terminal post-translational modification, resulting in targeting to the Golgi compartment en route to presynaptic sites. Three separate signals are required for this process. Two separate sequences in the amino-terminus of GAD65 are required for targeting of the cytosolic protein to Golgi membranes. In addition, palmitoylation of two cysteines is critical for trafficking of

GAD65 from Golgi to presynaptic SV clusters via a pathway which involves Rab5-positive endosomes and is shared with several other SV proteins (Kanaani et al., 2002 and 2003). The evidence that reduction in cellular cholesterol levels impairs targeting of GAD65 to presynaptic clusters led to propose that palmitoylation of GAD65 mediates its attachment to specialized membrane microdomains in the TGN, resulting in lateral segregation from non-palmitoylated proteins before the formation of axonal transport carriers (Kanaani et al., 2002 and 2004; See below).

Deletion and mutational analysis of synaptogyrin I, an abundant tetra-span SV protein distantly related to synaptophysin, has defined two sequence motifs responsible for its targeting to SVs (Zhao and Nonet, 2001). A single arginine in one of the cytoplasmic loops or a 38 amino acid sequence in the carboxy-terminal domain seems to be involved in the sorting of synaptogyrin I to SV precursors at the TGN and in endocytosis of the protein from the plasma membrane, respectively. Deletion of three tyrosines in the carboxy-terminal region is sufficient to cause the accumulation of synaptogyrin at the plasma membrane, thus implicating a tyrosine-mediated interaction with the AP2 complex in synaptogyrin endocytosis. Although the signal sequences identified in synaptogyrin I do not share homology with other SV protein-sorting sequences, it is interesting to note that the long, cytoplasmic carboxy-terminal domain of synaptophysin I appears to contain a signal for endocytosis (Linstedt and Kelly, 1991a).

The targeting of P-selectin to regulated secretory organelles of PC12 cells has been extensively investigated by Cutler and collaborators by expressing an HRP-tagged version of the protein in PC12 cells and following HRP activity through subcellular fractionation. Mutagenesis studies show that a tyrosine-based motif within the carboxy-terminal cytoplasmic tail is critical for trafficking of P-selectin to both SLMVs (at the level of early endosomes) and dense-core granules (at the level of the TGN), whereas additional determinants are responsible for the progression of P-selectin through

endosomal intermediates en route to SLMVs (Norcott et al., 1996; Blagoveshchenskaya et al., 1999b). These intermediate donor compartments correspond to both early and late endosomes. Interestingly, partially overlapping sequences located in the cytoplasmic domain on P-selectin are responsible for its BFA-sensitive sorting to SLMVs from different sites along the endocytotic pathway, namely either the late or early endosomal compartments. The analysis of double mutants, bearing deletions in both the sorting sequences, suggests that P-selectin travels to both SLMVs and lysosomes via the same two subsequent endosomal intermediates (Blagoveshchenskaya et al., 2000).

1.3.2 Microdomain-based sorting of SV proteins

Evidence for the formation of specialized domains in the plasma membrane come from the study of the role of cholesterol in SV formation and exocytosis.

Limited cholesterol depletion, which has only a weak effect on total endocytic activity, markedly affects both the size of the steady-state SLMV pool and SLMV biogenesis from the plasma membrane in PC12 cells (Thiele et al., 2000). Cholesterol is also essential for the formation of both regulated and constitutive secretory vesicles from the TGN in a neuroendocrine cell line (Wang et al., 2000).

Cholesterol-rich regions may undergo lateral phase separation from cholesterol-poor regions. In PC12 cells, SNARE-dependent exocytosis occurs at cholesterol-rich domains of the plasma membrane where syntaxin 1 and SNAP-25 are clustered (Chamberlain et al., 2001; Lang et al., 2001). Lowering of plasmalemmal cholesterol disperses t-SNARE hotspots and inhibits granule exocytosis.

Domains with high concentrations of cholesterol and sphingomyelin or glycosphingolipids, and associated proteins, termed 'lipid rafts', can be isolated as Triton-X-resistant fractions (Simons and Ikonen, 1997). The comparison of the lipid composition of rat brain synaptosomal plasma membranes (excluding SVs) with that of

purified SVs revealed that the composition of the two membranes is very similar, but with some striking differences (Hannah et al., 1999 and references within; Martin, 2000; Pfrieger, 2003). Gangliosides, which are present at high levels in the synaptosome plasma membrane, are almost completely absent in SVs. In contrast, SVs are enriched in acidic phospholipids and contain unusually high levels of cholesterol. Although the neuronal plasma membranes are themselves enriched in cholesterol (molar ratio of about 0.44), SVs show an extra-enrichment (molar ratio about 0.58). In addition, the accumulation of cholesterol in SVs is paralleled by a depletion, relative to the plasmalemma, in sphingolipids, with which cholesterol normally interacts. Therefore, SVs are generated by endocytosis of a cholesterol-rich (and sphingolipid-poor) domains of the plasma membrane.

This evidence prompted the investigation of whether one of the protein components of the SV is responsible for cholesterol binding and accumulation. The use of a radiolabelled photoactivatable analogue of cholesterol was exploited by Thiele et al. (2000) to search for cholesterol binding proteins associated with SLMVs of PC12 cells. This study allowed the identification of synaptophysin I as the major cholesterol-binding protein in SLMVs and brain SVs. This finding is of particular interest since synaptophysin I has been reported to be engaged in a detergent-resistant multimeric complex together with other SV proteins, namely VAMP2, SV2, synaptotagmin and the 39-kDa subunit of the vesicular proton pump (Bennett et al., 1992). Within this large complex synaptophysin I can interact directly with both VAMP2 (Calakos and Scheller, 1994, Edelmann et al., 1995, Washbourne et al., 1995, Pennuto et al., 2002) and the subunit of the proton pump (Thomas and Betz, 1990; Galli et al., 1996).

The establishment of a network of protein-protein interactions, together with the binding of synaptophysin I to cholesterol, may help to mediate the formation of membrane domains enriched in SV constituents from which budding of a new SV may

occur. The presence of synaptotagmin I, which binds the clathrin adaptor AP2, should ensure an efficient endocytosis of such preassembled modules. The hypothesis that synaptophysin I organizes lateral membrane domain in which SV proteins are selectively recruited while other membrane proteins are excluded is attractive (Figure 1.6).

Despite the conceptual analogy between the detergent-insoluble cholesterol/sphingolipid-based rafts and putative synaptophysin-cholesterol microdomains, two pieces of evidence argue against the view that SVs are raft-based organelles. First, although enriched in cholesterol, the SV membrane is low in sphingolipids, particularly in glycosphingolipids. Second, in contrast to raft proteins, synaptophysin is readily soluble in Triton-X-100 (Thiele et al., 2000). The latter result has been recently questioned by Gil et al. (2005) who identified a pool SV proteins (synaptophysin, VAMP2, Munc 18, synaptotagmin I and II) and t-SNAREs (syntaxin 1a and SNAP25) significantly associated with detergent-resistant membranes from rat brain synaptosomes.

Whatever the nature of the cholesterol-synaptophysin rafts, in order to clarify their function as sorting devices, it remains to be explained how t-SNAREs, which also are associated with plasma membrane domains with biophysical properties similar to the cholesterol-synaptophysin domains, are sorted away from SV proteins and therefore excluded from recycling SVs (Mitchell and Ryan, 2004, but see Walch-Solimena et al., 1995). Two conceivable explanations are that either the sorting machinery for the endocytotic step of SV recycling operates at sufficiently high fidelity to exclude internalization of abundant plasma membrane proteins such as t-SNAREs (i.e, lipid domains do not play a role in sorting during SV re-formation) or that t-SNARE themselves are restrained from entering sites of SV endocytosis owing to their

association with lipid domains distinct, yet biophysically indistinguishable, from SV protein-enriched domains.

Interestingly, at the plasma membrane and TGN, cholesterol depletion blocks the last steps of the budding process, characterized by the generation of negative curvature in the luminal membrane leaflet (Subtil et al., 1999; Wang et al., 2000). Cone-shaped lipids, such as cholesterol, favour negative membrane curvature. This is of particular importance in the case of SVs, where there could be a significant mismatch between the curvature present in the vesicles due to their small size and the curvature normally allowed by the phospholipids present in their membranes. In addition, association of cholesterol with oligomeric proteins, such as synaptophysin, might be important for the formation of the highly curved SV membrane.

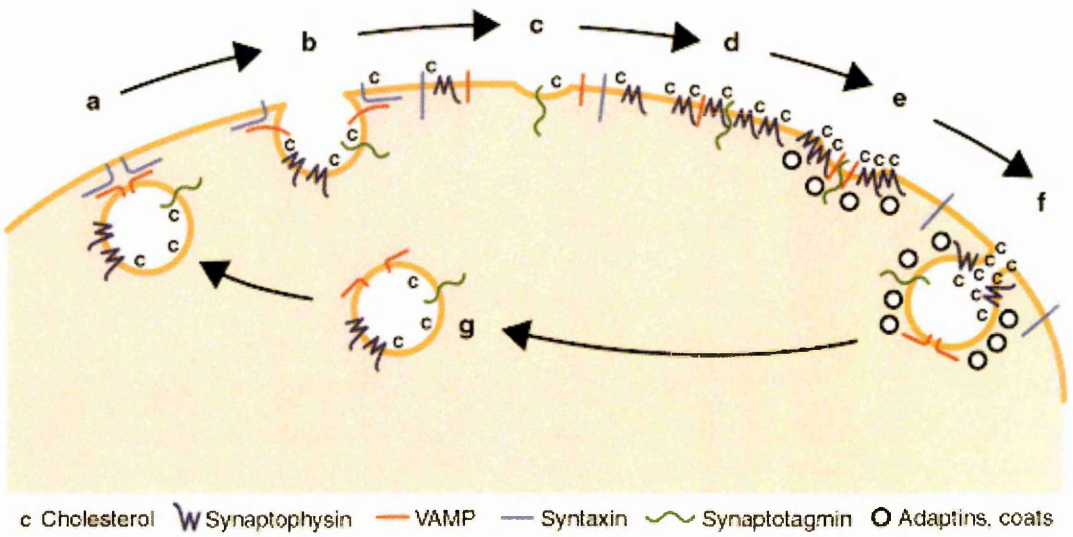

Figure 1.6. Cholesterol-rich microdomains participate in the SV life cycle. (Martin, 2000)
The SV is shown engaging in SNARE-protein-mediated exocytosis (**a**); the fusion pore then dilates (**b**) and the vesicle collapses into the presynaptic membrane (**c**), with dispersal of lipid and protein constituents (involving SNARE-complex disassembly). Reassembly of synaptic-vesicle constituents in the merged membrane (**d**) may involve lateral associations between cholesterol with sphingomyelin, synaptophysin and synaptotagmin, between synaptophysin and VAMP, and other protein–protein interactions. Recruitment of adaptins (such as AP-2) to cytoplasmic domains of synaptotagmin and other synaptic-vesicle proteins, and the recruitment of clathrin coat proteins (**e**), leads to invagination (**f**) and budding of a new synaptic vesicle (**g**).

1.3.3 Protein sorting at the TGN

Spatial segregation of cargoes is emerging as a crucial mechanism of polarized post-Golgi sorting. Indeed, recent evidence suggests that apical and basolateral cargoes segregate into large domains in the Golgi/TGN network structures even in non-polarized cells and exit in separate transport containers. Since these transport carriers do not intersect with endocytic structures, lateral segregation in the TGN appears to be the primary sorting event (Keller et al., 2001).

It is less clear whether this mechanism also operates in the sorting/transport of proteins destined to the same subcellular district (apical-axonal or basolateral-dendritic) although operating in different compartments (e.g. SV vs. endosomes or plasma membrane). A post-Golgi segregation step has been reported for apical proteins, then transported to the plasma membrane in different vesicular carriers (Jacob and Naim, 2001), while axonal proteins belonging to both SVs or plasma membrane have been shown to travel along the axon using the same (Ahmari et al. 2000) or distinct (Zhai et al., 2001) membrane carriers.

In epithelial cells certain apical proteins form detergent-insoluble sphingolipid-cholesterol rafts that are thought to function as platforms for apical-axonal delivery. These complexes are formed during protein processing in the Golgi apparatus and act in the TGN as sorting platforms for inclusion of protein cargo destined for delivery to the apical membrane (Simons and van Meer, 1988; Keller and Simons, 1998; Ledesma et al., 1998; Ledesma et al., 1999).

It is possible that, similarly to what has been observed for the sorting of apical and basolateral proteins in polarized epithelial cells, axonal or more specifically SV membrane constituents are pre-assembled into microdomains with specific lipid composition at the level of the TGN (Hannah et al., 1999). Although SVs are not generated by direct budding from the TGN, it has been suggested that selective

association of palmitoylated GAD65, the smaller isoform of the GABA-synthesizing enzyme glutamate decarboxylase, with specialized, cholesterol-rich, membrane microdomains in the TGN is a pre-requisite for GAD65 to enter the axonal trafficking route to presynaptic terminals, shared with other SV proteins (Kanaani et al., 2002 and 2003).

1.3.4 Stepwise assembly of SVs: a source of heterogeneity?

The sequential enrichment of proteins during the endo-exocytotic cycle of SVs implies that vesicles with different membrane composition can be present at the same time in the nerve terminals. This assumption contrasts with the concept that all the SVs from a particular neuron will have a uniform composition, and that they possess at least one member of each of the families of SV proteins (Sudhof et al., 1993, Takamori et al., 2000). However, it should be considered that, although biochemical analyses of isolated SVs demonstrate that their complement of proteins is largely similar, minor differences might have escaped detection. In addition, it is also likely that vesicles isolated by currently available procedures represent only a subset of the whole population (See Valtorta et al., 2001).

Purification of the total fraction of SVs from rat synaptosomes revealed that this preparation represents a mixture of at least two individual vesicular populations which differ in buoyant density and sedimentation. Remarkably, one of the vesicle pool carries some plasma membrane proteins (aminopeptidase, the neuro-specific isoform of the glucose transporter protein GLU3) in addition to specific SV proteins. The presence of these extra proteins appears to be accompanied by a decrease in the specific content of certain non-regulatory components of the SVs, such as the proton pump (Thoidis et al., 1998). It has been put forward that these vesicles may represent a hybrid of classical small SVs and the compartment responsible for their biogenesis. Hence, an intriguing

interpretation of these results is that one of the vesicle populations corresponds to mature SVs whereas the other contains 'maturing' SVs isolated during their cycling between donor membrane compartments.

The concept that a certain degree of heterogeneity is present in the SV population has been tested directly in the case of proteins of the synaptotagmin family. Analysis of SVs purified from adult rat brain cortex revealed a partially overlapped pattern of distribution of synaptotagmin I, II and IV in the same population of SVs (Osborne et al., 1999). Both synaptotagmin I and II are present on the same SV, and they heterodimerize efficiently in a calcium-dependent manner. In contrast, the distribution of synaptotagmin IV on SVs only partially overlaps with the distribution of synaptotagmin I and II. Moreover, in the subpopulation of SVs containing synaptotagmin I and IV, these two isoforms do not show a calcium-dependent interaction.

These results led to the proposal that the repertoire of synaptotagmin isoforms with different calcium binding features present on a single SV determines its exocytotic properties. This prediction was tested *in vivo* in Drosophila, where synaptotagmin IV bears an amino acid substitution which abolishes its ability to bind membranes in response to calcium influx. Synaptotagmin I and IV colocalize on the same population of SVs in Drosophila and the formation of hetero-oligomers decreases the ability of synaptotagmin I to penetrate membranes, resulting in downregulation of synaptic transmission owing to less efficient coupling between calcium entry and secretion (Littleton et al., 1999). Hence, changes in the relative abundance of distinct synaptotagmin isoforms on the same SV may modulate the calcium sensitivity of vesicle fusion.

The concept linking a certain level of variability in the composition of SVs to their exo-endocytotic properties, has been exploited to illustrate a process of 'competence

maturation' that might determine which recycling route is followed by SVs after neurotransmitter release (Valtorta et al., 2001). According to this model, the molecular make-up of SVs, at least in terms of accessory proteins, ultimately dictates whether the vesicle is competent for the kiss-and-run exo-endocytosis (i.e., without intermixing with the presynaptic plasma membrane), or incompetent, and therefore undergoes full fusion and recycling through coated vesicles. However, it should be considered that as well as relying on the specific composition of the vesicle, 'competence' might be determined by the location of the vesicles in the different functional pools, their interaction with nerve terminal proteins and post-translational modifications which modulate the properties of most of the SV proteins.

Clues as to the importance of protein composition of SVs in determining their recycling pathway comes from genetic studies which showed that spontaneous fusion rate was unchanged in the knockout of synaptotagmin I or complexins, proteins critical for evoked synchronous neurotransmitter release (Geppert et al., 1994; Reim et al., 2001). In contrast, spontaneous fusion rate was significantly reduced after deletion of the VAMP2 gene or completely abolished after genetic ablation of munc-18 or munc-13 isoforms (Schoch et al., 2001; Varoqueaux et al., 2002; Verhage et al., 2000).

Compelling evidence for distinct SV populations with different recycling properties within the same nerve terminal have been provided recently. Using dissociated hippocampal neurons, Sara et al. (2005) have shown that spontaneously recycling vesicles preferentially populate a 'reluctantly releasable' pool of limited size, which has limited cross-talk with vesicles of the activity-dependent recycling pool. Consistently, spontaneously endocytosed vesicles are more likely to be reused spontaneously, whereas they are only scarcely available for evoked release. Importantly, in striking contrast with wild type synapses, in synapses formed by VAMP2-knock out neurons SV endocytosed either spontaneously or upon stimulation undergo spontaneous exocytosis

at similar rates. Thus, in the absence of VAMP2, activity-dependent and spontaneous vesicle pools mix randomly. The fact that electron microscopic analysis does not reveal any obvious morphological or spatial difference between the two vesicle populations suggests that the functional segregation of these pools may be mediated by differences in their protein/lipid composition.

The existence at glutamatergic synapses of heterogeneous populations of SVs that can respond to different stimulations has been uncovered by estimating the readily releasable pool size using either hypertonic sucrose application or action potential trains (Moulder and Mennerick, 2005). Hypertonic stimulation estimates are five to six times greater than estimates derived from action potential trains commonly assumed to deplete the readily releasable pool. The reluctant vesicles might constitute a distinct pool which can be forced to recycle by applying strong depolarization and enhancing calcium influx. This reluctant pool of vesicles does not appear to exist in GABAergic neurons, suggesting that glutamate release may be subjected to a greater restraint than GABA release. The authors propose that the heterogeneity among glutamate vesicles from the same presynaptic terminal may underlie a new form of plasticity based on the use of functionally distinct vesicle pools (Moulder and Mennerick, 2005).

1.4 AXONAL TRANSPORT OF SYNAPTIC VESICLE PROTEINS

Axonal transport is essential to maintain the function, integrity and viability of neurons (Goldstein, 2003). Components of the different membrane compartments of the axon are transported from the cell body, the site of biosynthesis, to their destinations in the axon and synapses. In the past few years, several studies have visualized axonal transport in real time in living cells. The use of these optical approaches, together with sophisticated biochemical and ultrastructural analysis, has contributed a great deal to unravel the controversies which surround the composition and function of axonal vesicles. What are the sorting mechanisms and nature of the vesicular packages transported in the axon? Are functionally related proteins sorted together into single vesicles or complexes of vesicles? Although many issues remain unsolved, new paradigms begin to emerge.

The original concept that SVs are generated by direct budding from the TGN (See Zimmermann at al., 1993 for a discussion) was dismissed in the light of the key observation that membrane components accumulating on the proximal site of an axonal transport block could be classified either as vesiculotubular structures of 50-80 nm with some continuity with the axonal smooth endoplasmic reticulum, moving anterogradely, or large multivesicular bodies moving in the retrograde direction. No typical 50 nm spherical clear vesicles, the size and shape of SVs, were visible (Tsukita and Ishikawa, 1980). The lack of SVs in the axon implies that their components are transported down the axon on membrane precursors of mature SVs. SV are then generated by either a single or sequential steps along the endo-exocytic pathway.

It is unclear whether, in analogy to the traffic of newly synthesized SLMV components (Regnier-Vigoroux et al., 1991), SV proteins exploit membrane carriers mediating the constitutive axonal transport in order to be delivered to the site(s) where they are

assembled onto the final organelle. The visualization of GFP-tagged SV and plasma membrane proteins in living neurons allowed the identification of tubulovesicular organelles as a general transport machinery for newly synthesized proteins along the axon. The dynamics and morphology of SV precursors containing exogenous synaptophysin are largely similar to those displayed by the carriers used by functionally distinct plasma membrane proteins (Nakata et al., 1998). These vesicles move fast (~ 1 μm/s), bidirectionally, with frequent changes in direction, and are not labelled by endosomal tracers.

Although they defined a form of axonal transport shared by proteins destined to different compartments, Nakata et al., left unanswered the question as to whether all membrane proteins are transported by the same tubulovesicular carrier or whether they are sorted into different vesicles that are morphologically or behaviourally indistinguishable. Clues supporting the latter possibility come from the characterization of the motor proteins of the kinesin superfamily, which mediate anterograde transport. The neuro-specific kinesin KIF1A and the widely distributed isoform of the conventional mitochondrial motor KIF1Bβ, which share largely homologous tail domains, appear to be specific for the anterograde axonal transport of SV precursors, but not of presynaptic membrane proteins (Okada et al., 1995; Zhao et al., 2001). Consistently, mice lacking either KIF1A or KIF1Bβ exhibit lethal neurological phenotypes associated with a reduction in the density of SVs in the nerve terminals (Yonekawa et al., 1998; Zhao et al., 2001). In contrast, the conventional microtubule motor kinesin-I associates with the plasma membrane amyloid precursor protein (APP) and mediates its axonal transport on an organelle which also contain the neuronal membrane protein GAP43 but is devoid of SV proteins. As expected, mice lacking functional kinesin-I show a marked decrease in the axonal transport of both APP and GAP43, whereas the transport of SV proteins is normal (Kamal et al., 2000).

These data are corroborated by the use of dual-color video microscopy to track simultaneously the axonal transport of APP and synaptophysin (Kaether et al., 2000). These two proteins are sorted to, and transported in, different structures and at different net speeds. APP is transported in fast-moving (up to 4.5 μm/s) elongated tubes up to 10 μm in length, whereas synaptophysin is sorted to smaller tubulovesicular carriers. Furthermore, using morphological and biochemical approaches, Zhai et al. (2001) have provided evidence that a component of the active zone, the cytomatrix protein Piccolo, is transported and recruited into nascent synapses in ~80 nm dense core granulated vesicles together with other constituents of the active zone, including Bassoon, Syntaxin, SNAP-25 and N-cadherin, as well as chromogranin B. These granulated vesicles have been proposed to serve as a precursor for the presynapsic active zone assembly. Remarkably, none of the components of SVs is present in the active zone precursor vesicles.

These studies rule out the possibility that all membrane proteins in the synaptic region are transported on the same organelle to the synapse. It appears that membrane proteins destined to the different intracellular compartments are already sorted in the cell body to their specific cargo organelles. These organelles will be transported by their specific motors to their destinations.

The complexity of the axonal membrane traffic was further illustrated by Ahmari et al. (2000), who followed VAMP2 fused to GFP in real time in living neurons. The authors observed large fluorescent motile puncta comprising dense-core and pleiomorphic vesicles as well as tubulovesicular elements, containing SV proteins, a component of the endocytosis machinery (amphiphysin I) and calcium channels. Interestingly, nothing resembling a typical clear 50 nm SV was revealed by ultrastructural analysis. These fluorescent puncta, named 'transport packets' are recruited at the site of contact between an axon and a dendrite. Activity-dependent recycling of SVs is rapidly established at

new axodendritic sites (< 1 hour, see also Vardinon-Friedman et al., 2000). The dense core granulated vesicles found by Ahmari et al. (2000) in transport packets are likely to correspond to the active zone precursors described by Zhai et al., (2001).

Thus, it appears that membrane carriers of different nature and bearing functionally different cargoes are bundled together in a mobile unit containing many or all of the components required for construction of a presynaptic active zone (Figure 1.7). However, these studies do not clarify whether different SV proteins are delivered in the same precursor vesicle toward the site(s) where SVs are generated (i.e. either the axon or the synapse). Although the available data are somewhat contradictory, the picture emerging is consistent with the existence of multiple carriers involved in SV protein transport. It should also be considered that the situation might be significantly different in the case of either mature or immature neurons. Okada et al. (1995) reported that the axonal membrane carriers associate with KIF1A contain some, but not all, of the proteins common to SVs, such as synaptotagmin, synaptophysin, and Rab3A, but not SV2. This finding would imply that the incorporation of these proteins in the same SV, which seems to be mandatory for efficient neurotransmission, occurs at the synapse. In contrast, vesicles associated with the KIF1Bβ motor were found to contain both synaptotagmin, synaptophysin, and SV2 (Zhao et al., 2001), indicating that different motors are involved in the transport of an heterogeneous population of SV precursors with variable composition.

Interestingly, in immature neurons treated with BFA to induce tubulation of the Golgi apparatus, synaptophysin was redistributed to a tubular perikaryal-dendritic network positive for transferrin receptor, whereas other SV proteins (SV2, synaptotamgin, synaptogyrin, VAMP2, Rab3a) maintained their normal vesicular distribution (Mundigl et al., 1993). The heterogeneous distribution of SV proteins reported in this study further suggests that neurons, or at least developing neurons, contain a substantial

further suggests that neurons, or at least developing neurons, contain a substantial fraction of SV proteins which are not yet coassembled in SVs or SV precursors in the soma, consistent with a biogenesis of mature SVs distal to the TGN. In support of this concept, three different SV proteins, namely synaptophysin, SV2 and synaptotagmin, display a differential intracellular distribution when expressed in fibroblasts: synaptotagmin was found associated with the plasma membrane, synaptophysin with early endosomes and SV2 with an as yet unidentified organelle (Feany et al., 1993). Altogether these findings strengthen the idea that endo-exocytotic cycling of SV precursors, perhaps requiring passages through endosomal compartments, is essential to redistribute SV components to the same vesicle.

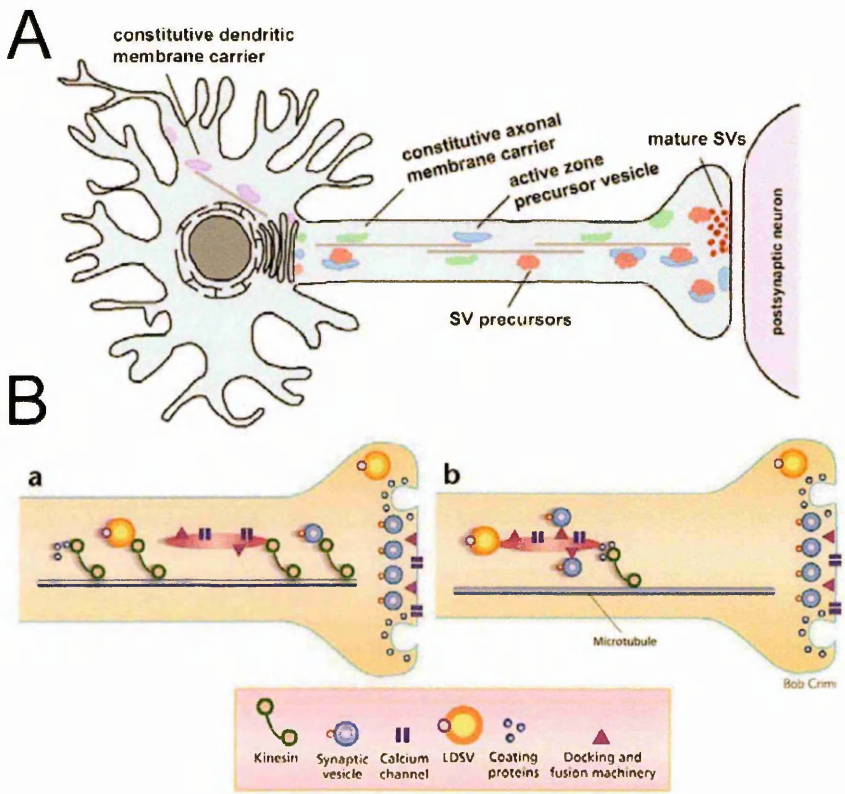

Figure 1.7. Trafficking routes of membrane proteins in neurons.
(A) After leaving the TGN, membrane proteins are targeted to either carriers mediating either constitutive secretion in axons or dendrites, or carriers specialized in the transport of active-zone and SV proteins. SV precursors assemble in clusters which appear to travel along the axon in association with active-zone precursor vesicles. At the nerve

(B) Transport of prefabricated prototerminals. Earlier models (**a**) predicted that the components of the nerve terminal (coating proteins, LDSVs, membranes containing presynaptic membrane proteins and synaptic vesicles) are transported down the axon in individual packets, with each packet recognized and transported by a different member of the kinesin family. The latest model (**b**) is that a pre-assembled complex of synaptic vesicle proteins, calcium channels, endocytotic machinery and LDSVs is transported as a unit at a speed close to that predicted for kinesin-mediated, microtubule-based transport (Roos and Kelly, 2000).

It is unclear at present whether the vesicular tubules associated with nascent synapses represent endosomal membranes or a repository for at least some components of the mature synapse. Evidences have been accumulating which support the existence of a partial overlap between the trafficking route of SV proteins along the axon and constitutive endosomal recycling:

1- Although the bulk of exogenous APP and synaptophysin molecules is sorted in distinct, highly mobile carriers which are not stained the by endosomal tracers, the two proteins are occasionally found to colocalize in slow or immobile short tubulovesicular/rounded structures that internalize either transferrin or dextran (Kaether et al., 2000).

2- Nakata et al. (1998) showed that most of the GFP-tagged synaptophysin, in a similar manner to plasma membrane proteins, is transported anterogradely by tubulovesicular carriers, supporting the possibility that SV proteins are directly transported from the TGN to the nerve terminal and then recycled rather then being targeted to axonal endosomes and then recycled to the axon (for a discussion see Kelly, 1993). However, exogenous synaptophysin is also associated with globular organelles which are labelled by endocytotic tracers and exhibit a preferential retrograde movement. The question arises as to whether synaptophysin in large globular endosomes is again recruited for the biogenesis of SVs, or it is en route to be degraded in the cell body.

3- Along the axon syntaxin 13, a recycling endosome marker, is associated with two classes of organelles: round-oval, stationary structures, and fast-moving tubulovesicular elements. Membrane dynamics suggestive of fusion and budding events between the two classes of organelles were reported, consistent with the hypothesis that the mobile tubulovesicular endosomes might shuttle cargo to and from the stationary endosomes. Both types of organelles do not contain a significant amount of SV proteins, although colocalization is occasionally observed (Prekeris et al., 1999). It should be noted anyway that the identification of transporting vesicles by immunocytochemistry of fixed samples is often hampered by the small amount of membrane proteins being transported relative to the fraction that has already reached the target (Nakata et al., 1998 for discussion, but see Ahmari et al., 2000). The use of dual-color videomicroscopy to monitor in real time the axonal trafficking of fluorescent chimeras of both SV and endosomal proteins, in combination with biochemical studies, will be critical to unequivocally estimate the molecular identity of the tubulovesicular structures delivering SV proteins to the synapse.

1.5 SYNAPTIC VESICLE RECYCLING IN DEVELOPING NEURONS

1.5.1 Mechanisms of neurotransmitter secretion from developing axons

Neurotransmitter release appears early in the developing embryo and might play a role in morphogenesis, neuronal communication and axonal navigation. Several lines of evidence indicate that neurons are endowed with a functional apparatus for neurotransmitter release prior to the establishment of the synaptic contacts. The rapidity of synapse formation (< 1 h, Vardinon-Friedman et al., 2000) suggests that neurons may have already acquired the appropriate machinery of neurotransmitter release before encountering the target cell.

Both spontaneous and evoked release of various neurotransmitters from developing axons has been reported from a number of preparations (see Matteoli et al., 2004 for references). Neurotransmitter release is restricted to the distal axon and growth cones, whereas is not detected at the level of the soma. In particular, evoked release is detected only at selected sites along the axon, and in the growth cone, which displays the most efficient excitation-secretion coupling (Young and Poo, 1983; Hume et al., 1983). A gradient of secretory activity along the axon, which is shaped by developmentally regulated mechanisms, has been described in Xenopus spinal cord neurons (Antonov et al., 1999). At early stages of axonal growth, neurotransmitter release takes place along the axon as well as the growth cone, whereas at later stages it follows a proximodistal gradient, with the highest level at the growth cone. These results point to the presence of regulatory elements underlying the preferential localization of the machinery for neurosecretion at the growth cone.

Whether at these stages SVs are needed for neurotransmitter release is unknown. Neurotransmitters might be stored and released from cellular compartments other than

SVs, such as the endoplasmic reticulum or the cytoplasm. It was initially hypothesized that the release of neurotransmitters during neuronal development occurs as a consequence of the incorporation of new membrane during neurite extension (Young and Poo, 1983). This conclusion was suggested by the pulsatile nature of both spontaneous and evoked release in developing neurons relative to the kinetics of release measured at synapses. Although this hypothesis has not been completely ruled out, the presence of vesicles bearing SV proteins in young neurons (Fletcher et al., 1991), together with the evidence that SVs are not engaged in neurite extension (Leoni et al., 1999), argue against a role for membrane carriers devoted to axon outgrowth in this immature form of neurotransmitter release.

The involvement of vesicles in neurotransmitter release along the axons has been inferred by its sensitivity to BFA, which does not affect SV recycling at mature synapses (Zakhareko et al., 1999). Since the effect of BFA on neurotransmitter secretion was observed even in transected axons, it is likely to depend on the inhibition of the ARF-mediated local recycling of vesicles through axonal endosomes rather than of vesicle budding from the TGN. The existence of a vesicular mechanism underlying neurotransmitter secretion along the axon is also supported by the increase of either GABA (Gao and van den Pol, 2000) or acetylcholine (Zakhareko et al., 1999) release by α-latrotoxin, which enhances SV exocytosis at the synapse (Valtorta et al., 1988). In addition, the secretion of acetylcholine can be triggered by a hypertonic solution, suggesting the presence of a pool of fusion-competent vesicles docked at the axolemma (Zakhareko et al., 1999).

Important clues as to the mechanisms underlying the release of neurotransmitters from developing neurons come from the study of quantal neurotransmitter secretion from either non-neuronal cells loaded with acetylcholine or Xenopus spinal neurons manipulated into contact with a muscle cell used as a detector (Dan and Poo, 1992;

Girod et al., 1995). Both spontaneous and evoked exocytosis are reported to occur from myocytes and fibroblasts loaded with acetylcholine, which reflect the Ca^{2+}-dependent exocytosis of acetylcholine-filled vesicles (Dan and Poo, 1992; Morimoto et al., 1995). Compared with the regulated neurotransmitter secretion from presynaptic nerve terminals of the neuromuscular junction, excitation-secretion coupling in fibroblasts and myocytes is significantly weaker, but surprisingly close to that found at developing neuromuscular synapses in culture (Evers et al., 1989; Girod et al., 1995). The kinetics with which the neurotransmitter packets are discharged from either non-neuronal cells or developing neurons are comparable, although the speed of secretion in non-neuronal cells is slower and more irregular.

The striking similarity of the size and distribution of neuronal miniature endplate currents with those detected in non-neuronal cells supports the notion that SVs in developing neurons are derived from a similar population of vesicles used for constitutive secretion in all cell types (Girod et al., 1995). In developing neuromuscular junctions the quantal size varies over a wide range, resulting in a skewed distribution of miniature endplate currents detected in the postsynaptic muscle cell (Evers et al., 1989). A high variability in the amplitudes of quantal transmission has also been found at nascent central synapses (Liu and Tsien, 1995).

A skewed amplitude distribution may result from the accumulation of either different concentrations of neurotransmitter in vesicles of similar size or comparable concentrations in vesicles of different size. The presence of pleiomorphic vesicles along isolated axons and nascent synapses (Kraszewski et al., 1995; Ahmari et al., 2000) is consistent with the latter possibility. Thus, neurotransmitter secretion in developing neurons may be sustained by a population of SV precursors of variable size and may originate from a ubiquitous pathway used for constitutive secretion and membrane trafficking in non-neuronal cells which acquires specific regulatory properties during

neuronal differentiation. Consistent with this model, isolated neurons exhibit a limited spontaneous quantal neurotransmitter secretion, and marked increase in the frequency of secretion can be induced by specific contact with a myocyte (Xie and Poo, 1986; Sun and Poo, 1987). Thus, it is conceivable that the presence of a neurosecretory apparatus in developing neurons has the direct functional consequence of rapidly establishing synaptic transmission at the nascent synapse (See Ahmari et al., 2000). However, in the light of these results the striking similarity between the parameters of spontaneous and evoked neurotransmitter secretion measured in either the axon or nerve terminal using a myocyte manipulated into contact with the axon (Sun and Poo, 1987; Zakharenko et al., 1999) might reflect the induction of the program of synaptic maturation rather than the genuine properties of neurotransmitter release from isolated processes.

It appears that spontaneous neurotransmitter release is inhibited in isolated axons and that such inhibition is reduced upon contact with the postsynaptic cell. Thus, SV recycling in developing neurons is actually under the control of regulatory mechanisms rather than being functionally associated with an housekeeping form of constitutive axonal recycling (See Matteoli et al., 1992). At present, two signaling cascades modulating neurotransmitter secretion from axonal growth cones have been described. Evoked GABA secretion from growth cones is stimulated by externally applied acetylcholine via protein kinase C activation (Girod et al., 1995; Gao and van den Pol, 2000). In addition, genetic manipulation of cAMP-dependent pathways in Drosophila provides evidence for a role of cAMP regulation in acetylcholine release from the growth cones (Yao et al., 2000).

Beside the release of neurotransmitters by vesicle exocytosis, nonvesicular modes of neurotransmitter secretion have been observed in several preparations and are generally thought to occur via the reversion of transporters (Gaspary et al., 1998) or through exchangers (Warr et al., 1999). Reversion of transporters is not involved in a

nonconventional, Ca^{2+}- and SNARE-independent, mode of GABA and glutamate release identified in synaptically silent CA1 pyramidal neurons during the perinatal period. This paracrine mode of release, which generates large slow currents, is restricted to an early developmental stage, and provides a substantial components of the communication between immature neurons, at least in this neuronal population (Demarque et al., 2002). The persistence of this early slow current in Munc18-1-deficient mice, in which vesicular release has been completely abolished (Verhage et al., 2000), indicates that the release has a nonvesicular origin or arises from vesicles that do not use the conventional SNARE machinery for exocytosis.

1.5.2 Properties of synaptic vesicle recycling in growing axons

The expression of mRNAs encoding SV proteins is rapidly switched on in young post-mitotic neurons prior to neurite extension and synapse formation (Marazzi and Buckely, 1993). Before synaptogenesis, vesicles containing SV proteins concentrate in the axon and upon contact with the postsynaptic cell become clustered at synaptic sites (Fletcher et al., 1991). However, the relationship between the vesicles recycling along the axon and genuine SVs in the nerve terminal remains to be established (i.e., it is unknown whether the composition of SVs in immature neurons is at least partially different from the composition of mature SVs present at the synapse). The absence of classical small SVs in the transport packets described by Ahmari et al. (2000) indicates that pleiomorphic precursor vesicles are likely to be involved in the trafficking of SV proteins along the axons (See also Matteoli et al., 1992; Kraszewski et al., 1995).

Although the contribution of SVs to neurotransmitter secretion in developing neurons remains to be determined, vesicles bearing SV antigens have been shown to recycle along the axon. A seminal set of experiments has been performed following the internalization of an antibody directed against the lumenal domain of SV-specific

protein synaptotagmin I (Matteoli et al., 1992; Kraszewski et al., 1995). Using this tool Matteoli et al. (1992) showed that clusters of SVs (~10-20 SVs per cluster) undergo multiple cycles of exo-endocytosis along the isolated axon of developing neurons. Since labelled SVs are eventually found at synapses formed days after the uptake of the antibody, it appears that following repetitive exchanges of components with the axolemma SVs are recruited to the presynaptic terminals. As observed at the mature synapse (Betz and Henkel, 1994), SV clusters are maintained through a phosphorylation-dependent mechanism, since the inhibition of phosphatase activity causes disruption of vesicle clusters (Kraszewski et al., 1995). SV clusters move in anterograde and retrograde directions in the axon and, upon contact with the target neuron, become immobilized into the newly forming presynaptic terminal (Kraszewski et al., 1995; Ahmari et al., 2000). Interestingly, neurotransmitter release along the axon is markedly reduced after the formation of synapses, suggesting that SV precursors which were available for exocytosis at extrasynaptic regions before synapse formation have been recruited at the nascent synapse (Chow and Poo, 1985). The mechanisms underlying the recruitment and immobilization of SV packets into the nascent synapse are still unknown.

Recycling of SVs in isolated axons is Ca^{2+}-dependent and poorly sensitive to depolarization, although high levels of constitutive exo-endocytosis are detected. Importantly, the formation of synaptic contacts correlates with a downregulation of the basal rate of SV recycling, suggesting the strengthening of a mechanism which blocks constitutive exocytosis (Kraszewski et al.,1995). In addition, *in vivo* imaging of SV precursor clusters combined to the use of FM dyes to visualize activity-dependent endocytotic processes reveals that the mobile clusters in isolated axons do not undergo evoked recycling, whereas SVs located at stable synaptic puncta show strong activity-dependent FM staining (Ahmari et al., 2000). Together, these results indicate that in the

axon SV recycle along a constitutive pathway of secretion which is scarcely sensitive to depolarization, and enter an activity-regulated pathway as recruited at nascent synapses. The presence of an inhibitory mechanism of spontaneous recycling associated with a Ca^{2+}-regulated enhancement of SV exocytosis are needed to gain an efficient excitation-secretion coupling at the synapse (Popov and Poo, 1993).

A further indication that the secretion of neurotransmitter from developing axons might be ontogenetically related to the constitutive endosomal recycling pathway is provided by the selective effects of BFA on neurotransmitter secretion in young but not mature neurons (Zakhareko et al., 1999). Therefore, in developing neurons an ARF/AP3-mediated process of vesicle re-formation from endosomes similar to that described in neuroendocrine cells (Faundez et al., 1997 and 1998) takes place. Such process is later substituted by a dynamin/AP2 mechanism of vesicle generation directly from the plasma membrane (De Camilli and Takei, 1996). Therefore, the machinery involved in SV recycling in developing neurons is at least partially different from that operating at the mature synapse.

The differential expression of vesicle components might also underlie the presence of distinct regulatory mechanisms of SV exocytosis during neuronal development. Indeed, it appears that before synapse formation the exocytotic machinery operating in isolated axons utilizes a tetanus-toxin-resistant isoform of VAMP2 which is substituted by a VAMP2-dependent exocytotic pathway as synapses form (Verderio et al., 1999). This change in the composition of the exocytotic machinery correlates with a change in the recycling properties of SVs, as shown by the reduction in the rate of spontaneous exocytosis following the establishment of synaptic contacts.

Thus, during neuronal development changes in the expression of SV components and in the pathway of SV biogenesis are needed to generate a pool of homogeneous clear small vesicles, which are typically associated with the presynaptic terminals, from the

pleiomorphic vesicle population found in the isolated axons. It has been proposed that the multiple cycles of exo-endocytosis undertaken by vesicle precursors along the axon might play a role in the morphological and functional maturation of SVs, allowing the redistribution of membrane components between the SV and the axolemma (Matteoli et al., 2004; Figure 1.8).

This hypothesis has been corroborated by the study of axonal membrane trafficking in Drosophila neurons lacking sec5, an essential component of the exocyst complex implicated in trafficking to the cell surface. This mutation impairs membrane addition of newly synthesized proteins whereas SV fusion at the synapse is not affected. Importantly, newly synthesized synaptotagmin, though transported down the axon, does not enter the pool of mature SVs clustered at synaptic boutons and remains in the axon or is sent retrogradely back to the soma. Therefore, it seems that vesicles bearing SV antigens which can not fuse with the plasma membrane in the absence of a functional exocyst complex, are not matured into SVs (Murthy et al., 2003).

It is also conceivable that during repetitive cycles of SV fusion the plasma membrane becomes enriched or depleted in specific components and that these changes might underlie important neuronal functions (See Matteoli et al., 2004). The presence in growth cone filopodia of vesicles bearing SV proteins which can be stimulated to fuse with the plasma membrane suggests that the composition of individual filopodia can be rapidly altered in response to external stimuli, such as the contact with a target, in order to express at the surface a set of molecules involved in axon guidance and synapse formation (Sabo and McAllister, 2003). Consistent with this view, recycling of SV precursors in isolated axons mediates the insertion of glutamate AMPA receptors in the growth cone membranes (Schenk et al., 2003) and the activation of axonal AMPA receptors modulates filopodia dynamics (Chang and De Camilli, 2001; De Paola et al.,

De Paola et al., 2003) and growth cone motility (Schenk et al., 2003), and triggers SV exocytosis through a positive feedback loop (Schenk et al., 2005).

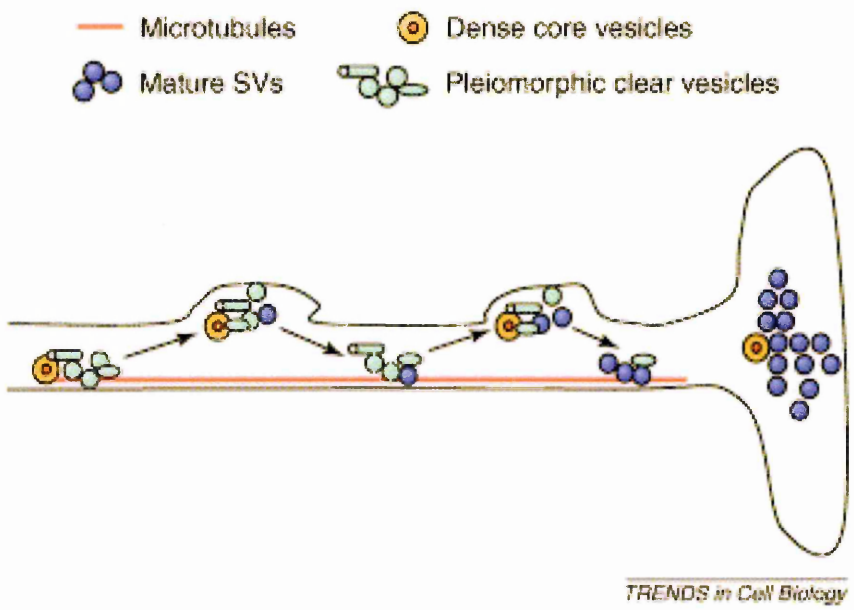

Figure 1.8. SV recycling in developing axons (Matteoli et al., 2004).
Pleiomorphic SV precursors undergo repetitive cycles of fusion with the plasma membrane leading to the generation of mature SVs, which are then recruited to the nascent synapse.

1.6 SYNAPTOPHYSIN: STILL LOOKING FOR A ROLE

Synaptophysin (Syp) was the first SV protein to be cloned. After the initial excitement raised by its discovery, the study of the role of Syp in SV exo-endocytosis has been somewhat neglected, probably because of the absence of an overt phenotype in Syp knocked-out animals. However, a large body of experimental data both *in vitro* and *in vivo* indicate that Syp (alone or in association with homologous proteins) is involved in multiple, important aspects of SV exo-endocytosis, including regulation of SNARE assembly into the fusion core complex, formation of the fusion pore initiating neurotransmitter release, activation of SV endocytosis and SV biogenesis (Valtorta et al., 2004).

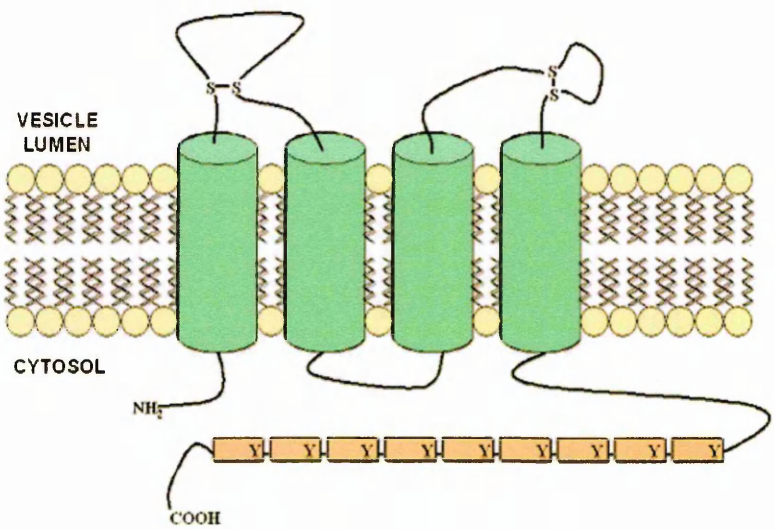

Figure 1.9. The tertiary structure of SypI.
Schematic model of the tertiary structure of Syp, consisting of a cytoplasmic NH2-terminus, four membrane-spanning domains, two hydrophilic intravesicular loops each containing a disulfide bond and a long COOH-terminal tail containing nine repeats which start with a tyrosine residue.

1.6.1 General features

Synaptophysins are a family of integral membrane proteins, whose first member, p38 or synaptophysin I (SypI), was identified in the 80s as a major and specific component of SVs (Jahn et al., 1985; Wiedenmann and Franke, 1985; Buckley et al., 1987; Leube et al., 1987). SypI is a 38,000 dalton glycoprotein that spans the membrane of the vesicle four times, with both the amino- and the carboxy-termini on the cytosolic side of the SV membrane (Figure 1.9). The four hydrophobic transmembrane domains are separated by two short intravesicular hydrophilic loops. Both intravesicular loops of the molecule are circularized by a disulfide bond. The first loop is N-glycosylated. This post-translational modification is responsible for the heterogeneity of the molecular weight of the protein (Navone et al., 1986), and seems not to be required for the correct targeting of the protein to small cytoplasmic vesicles in non neuroendocrine cells (Leube et al., 1989).

The 90 amino acid carboxy-terminal tail is the prominent cytosolic portion of the protein and is characterized by an unusual amino-acidic composition, which includes ten copies of proline and glycine rich pentapeptide repeats, nine of which begin with a tyrosine residue. The presence of numerous proline and glycine residues is supposed to confer to the tail a rigid, non-α-helical structure (Südhof et al., 1987a and b). This region contains an internalization signal that allows the protein to be recruited on vesicles by endocytosis from the plasma membrane (Linstedt and Kelly, 1991a).

The carboxy-terminal domain is phosphorylated on serine and tyrosine residues by Ca^{2+}-calmodulin dependent protein kinase II and $pp60^{c\text{-}src}$, respectively (Pang et al., 1988; Barnekow et al., 1990; Rubenstein et al., 1993). Recently, phosphorylation of SypI has been correlated with inhibition of neurotransmitter release and synaptic plasticity (Mullany and Lynch, 1998; Asermely et al., 1999). Interestingly, a strong increase of SypI and $pp60^{c\text{-}src}$ interaction has been found to correlate with synaptic activity enhancement during spatial learning and memory formation in the hippocampus

(Zhao et al., 2000). However, evidence for a direct role of SypI phosphorylation on neuroexocytosis is still missing. Furthermore, SypI has been shown to bind Ca^{2+} via its cytosolic carboxy-terminal tail (Wiedenmann and Franke, 1985; Rehm et al., 1986). However, a more detailed characterization of this property indicated that the Ca^{2+} - binding ability of SypI is negligible at $[Ca^{2+}]_i$ up to 10^{-4} M (Brose et al., 1992).

SypI forms non covalent homo-oligomers, from dimers to hexamers, *in vitro* (Jahn et al., 1985; Rehm et al., 1986; Thomas et al., 1988; Johnston and Südhof, 1990) as well as *in vivo* (Pennuto et al., 2002). Beside interacting with other Syp molecules to form homo-oligomers, Syp has been found to form hetero-oligomers *in vitro* with various nerve terminal proteins, that include the v-SNARE vesicle-associated membrane protein 2/synaptobrevin II (VAMP2) (Calakos and Scheller, 1994; Washbourne et al., 1995; Edelmann et al., 1995), the vesicular proton pump V-ATPase (also termed physophilin) (Thomas and Betz, 1990; Galli et al., 1996; Carrion-Velasquez et al., 1998), myosin V (Prekeris and Terrian, 1997), dynamin I (Daly et al., 2000; Daly and Ziff, 2002), and the adaptor AP-1 (Horikawa et al., 2002). The functional roles of some of these interactions are discussed below.

1.6.2 Cellular and subcellular distribution

SypI is expressed in both the central and peripheral nervous system, with a distribution similar to that of synapsin I (Navone et al., 1986; Wiedemann et al., 1986). SypI is also expressed in endocrine and neuroendocrine tissues, such as the adrenal medulla, and in neuroendocrine tumors and tumor-derived cell lines, like PC12 (Navone et al., 1986; Obendorf et al., 1988; Wiedemann et al., 1986; Vetter et al., 1989). Indeed, SypI is used as antigenic marker in tumors of neuroendocrine type (Wiedemann et al., 1986).

At the subcellular level, SypI is found in association with SVs in neurons and with SLMVs in PC12 cells (Navone et al., 1986; Lah and Burry, 1993). Stimulation of SV

exocytosis by α-Ltx results in the appearance of the protein in the plasma membrane (Valtorta et al., 1988). Moreover, upon treatment of neuronal cells with the toxin in the absence of extracellular Ca^{2+}, a condition in which endocytosis is impaired, as well as upon depletion of SVs by prolonged high-frequency stimulation, SypI diffuses throughout the plasma membrane (Torri-Tarelli et al., 1990 and 1992).

Localization of SypI in large dense-core granules (LDCG) and secretory granules of endocrine cells is debated. Indeed, although SypI has been reported to be absent from these organelles by some authors (Wiedenmann and Franke, 1985; Navone et al., 1986; Marxen et al., 1997), the presence of small amounts of SypI in bovine chromaffin granules of the adrenal medulla and in LDCGs of PC12 has been documented by others (Obendorf et al., 1988; Lah and Burry, 1993). This discrepancy probably comes from the limited concentration of the protein present on LDCGs relative to the total fraction associated with SVs. The expression of SypI throughout the nervous and the neuroendocrine system and the presence of the protein on the organelles associated with to regulated secretion of both classical and peptidic neurotransmitters suggest a general function for SypI in neuroexocytosis.

1.6.3 Synaptophysin I is a member of a highly conserved family of proteins

SypI is highly conserved throughout evolution. SypI orthologs are present from invertebrates to mammals, although they are not found in yeast. Global alignment of SypI vertebrate orthologs reveals overall amino acid conservation, the most divergent regions being the second intravesicular loop and the carboxy-terminal tail (Hübner et al., 2002). When the C. elegans ortholog is included in the analysis, the four hydrophobic transmembrane domains, the short linker sequence between the second and third transmembrane regions and, to a lower degree, the first intravesicular loop result to be the most conserved sequences of the protein. Strikingly, a positively charged residue (Lys in vertebrates, Arg in the nematode) of the first transmembrane region, which has been shown to be critical for the correct topogenesis of the protein (Leube, 1995), is conserved in all the orthologs.

The C. elegans SypI presents an unusually long amino-terminal with hydrophobic features, and a particularly short carboxy-terminal tail. Paired cysteines engaged in the formation of the two disulfide bonds are conserved in all the orthologs in the first intravesicular loop, whereas they are absent in the C. elegans SypI in the second intravesicular loop. Interestingly, both the known and putative sites of post-translational modifications are highly conserved in all the vertebrate orthologs but not in C. elegans. The N-glycosylation site (Asn) in the first intravesicular loop of vertebrates is indeed substituted to Asp in the nematode, thus preserving the structural character of the amino acid, whereas the putative phosphorylation site (Thr) in the carboxy-terminal sequence of vertebrate SypI is changed to Val in the nematode. The second and third phosphorylation sites (Tyr), which are evolutionary conserved in vertebrates, are not included in the short carboxy-terminal tail of the nematode ortholog. The absence of

post-translational modification sites in the C. elegans ortholog might provide interesting clues as to the functional evolution of SypI.

Proteins of the synaptophysin family include both neuronal and non neuronal members. Indeed, the search for SypI isoforms has led to the discovery of not only a brain specific isoform named synaptoporin, but also of the ubiquitous isoform named pantophysin and the skeletal muscle-specific isoform mitsugumin 29. Interestingly, the expression of the individual members of the synaptophysin family is not mutually exclusive at both a cellular and subcellular level.

Amino acid sequence comparison between SypI and synaptoporin, pantophysin and mitsugumin 29 reveals 66% identity with the former, and 47% identity with the latters (Hübner et al., 2002). Moreover, all the proteins share several features, like exon-intron gene organization, membrane topology, N-glycosylation, the presence of a disulfide-bond at the level of each intravesicular loop, the ability to form homo-oligomers, and a high degree of conservation through evolution.

Synaptoporin, also called synaptophysin II (SypII) was discovered in 1990 (Knaus et al., 1990). SypII is a 37,000 dalton protein. It lacks the cytosolic amino-terminal domain and displays 58% amino acid identity to SypI. The regions of highest homology between SypI and SypII are the transmembrane segments and the intravesicular loops. The most divergent domain is the carboxy-terminal tail, although short peptide motives are shared by the proteins. The tail of SypII is shorter, serine-rich and is characterized by the presence of five highly degenerate pentapeptides starting with a tyrosine residue. The protein, however, does not seem to be tyrosine phosphorylated (Janz et al., 1999).

If the carboxy-terminal domain plays a regulatory, rather than structural, role in modulating protein function, the different motifs might mirror functional specialization of the two isoforms. At variance, the high degree of similarity between SypI and SypII might predict similar or even redundant roles for the proteins in neuroexocytosis.

However, the distribution of the two isoforms is only partially overlapping. In general, SypI displays a more widespread distribution compared to SypII. For instance, in the hippocampus, SypII is not expressed in the stratum oriens of the CA3 region and in the molecular layer of the dentate gyrus. SypII, but not SypI, shows a rostro-caudal gradient in the cerebellar cortex, while very little amounts of the protein are detectable in the granular layer (Fykse et al., 1993). In those regions of the nervous system where both SypI and SypII are expressed, they localize on the same SV. SypII also forms homo-oligomers, but hetero-oligomers between the two isoforms have never been described.

Pantophysin is the only ubiquitously expressed member of the family identified so far (Haass et al., 1996). Contrary to the brain isoforms, pantophysin lacks a carboxy-terminal tail. The protein is found in association with vesicles belonging to the constitutive transport. However, pantophysin is also expressed in the brain, where it colocalizes with SypI and SypII (Haass et al., 1996). Intriguingly, despite its ubiquitous distribution, pantophysin seems to be enriched in tissues which display pronounced membrane traffic, e.g. liver and adipose tissue, suggesting a possible role of the protein in these processes. In adipocytes, pantophysin was found in association with insulin-responsive vesicles positive for the GLUT4 glucose transporter (Windoffer et al., 1999; Brooks et al., 2000).

Mitsugumin 29 has been recently identified as an abundant component of the skeletal muscle, although it is also present at low levels in renal tubular cells (Shimuta et al., 1998). Mitsugumin 29 is a specific and essential component of the triad junction, the site where the depolarization signal is converted into Ca^{2+} release from the sarcoplasmic reticulum.

A further degree of complexity comes from the existence of other protein families which display homologous structure to Syp. Two classes of multimeric proteins showing similar membrane topology and localization to synaptophysins have been

described: the synaptogyrins and the secretory carrier-associated membrane proteins (SCAMPs).

The first member of the synaptogyrin family was initially termed p29 (Baumert et al., 1990) and subsequently renamed synaptogyrin I (Stenius et al., 1995). Other members of the synaptogyrin family include synaptogyrinII/cellugyrin, which is expressed in all tissues but the brain, whereas synaptogyrin I is restricted to neuronal and neuroendocrine tissues, where it associates with SVs. The synaptogyrins are highly homologous to each other, and, although they show a strong molecular and structural similarity to synaptophysins, the two classes of proteins share only a 10-15% identity in the primary structure (Janz and Südhof, 1998).

Five members of the SCAMP family have been identified so far (Fernández-Chacon and Südhof, 2000). Albeit these proteins are ubiquitously expressed, a certain degree of tissue-specificity has been reported for SCAMP1, the most abundant variant of the family, which is enriched in the brain in association with SVs, for SCAMP5, whose expression is restricted to the nervous system, and SCAMP2, that is abundantly expressed in polymorphonuclear leukocytes. The presence of SCAMPs in all post-Golgi carrier vesicles, including the recycling transport vesicles, and in secretory organelles, including SVs and secretory granules, is suggestive for a general function in vesicle trafficking.

The existence of ubiquitous and tissue-specific members of the synaptophysin family and, in addition, the presence of other protein families which display similar structure and distribution may indicate that these proteins act in concert to exert similar functions in different tissues. Despite the low similarity in the primary structure, all tetraspan membrane proteins have been found to be abundant components of organelles devoted to constitutive or regulated vesicle trafficking (Hübner et al., 2002). Thus, the possibility arises that such a molecular structure underlies a general function. Moreover,

tetraspan membrane proteins might have been originated from a common ancestor, and then diverged to acquire specialized functions depending on their tissue or subcellular distribution (evolutionary divergence). Alternatively, this protein structure might have been originated independently in different trafficking pathways in order to exert a similar function (evolutionary convergence).

1.6.4 Synaptophysin I is involved in the regulation of neurotransmitter release

The first evidence pointing to the involvement of SypI in the regulation of neurotransmitter release came from experiments in which Ca^{2+}-dependent glutamate release was reconstituted in Xenopus laevis oocytes via injection of total rat cerebellar mRNA. In this system, co-injection of either antisense oligonucleotides or antibodies against SypI resulted in a marked reduction of Ca^{2+}-induced glutamate release (Alder et al., 1992a). These findings have been recently confirmed for other neurotransmitters (Shibaguchi et al., 2000).

Loading of motor neurons with antibodies against SypI by injection either in early blastomeres of Xenopus embryos or directly into cultured neurons strongly reduced the frequency of spontaneous synaptic currents without affecting their mean amplitude. In those preparations, the impulse-evoked neurotransmitter release was strongly reduced in amplitude and in many cases the complete loss of evoked synaptic currents was observed (Alder et al., 1992b). Consistently, overexpression of SypI in Xenopus embryos resulted in a strong increase in the frequency, but not in the amplitude, of spontaneous synaptic currents, and in an increased amplitude and reduced delay of onset of evoked synaptic currents (Alder et al., 1995). Altogether, these results suggest that SypI is not implicated in determining the quantal size of release, but is involved in the late steps of exocytosis.

Apparently contradictory data were obtained by Sugita et al. (1999) in the neuroendocrine cell line PC12 engineered to secrete human growth hormone. In this cell line, overexpression of Syp decreased to some extent growth hormone secretion, thus pointing to a negative role for Syp in exocytosis. However, it should be pointed out that growth hormone is packaged in large dense-core granules, and not in SVs, and that the presence of Syp in the membrane of large dense-core granules is a debated matter (see above).

But at which step(s) of the SV cycle does Syp act? And what is the direct role of the protein in membrane fusion? In order to answer these questions, in the same period two different groups knocked out the gene encoding Syp (Eshkind and Leube, 1995; McMahon et al., 1996). Unfortunately, the strategy to clarify Syp function by deleting the gene was not successful, since Syp knock out mice did not show an overt phenotype. The mice are viable and fertile, no structural alterations in the nervous system are visible and many parameters of synaptic transmission are unchanged, including short-term and long-term potentiations.

The absence of an obvious phenotype may be explained either by some degree of redundancy in Syp function, attributable to the presence of Syp homologues that can compensate for the absence of Syp, or to the lack of competition, during synaptogenesis, between Syp-positive and negative neurons. Consistent with the former possibility, a careful examination of the Syp knock-out phenotype has led to the discovery that in these mice retinal rod photoreceptors, which normally lack detectable levels of synaptoporin and express Syp at high concentration, show several defects in cell membrane organisation and a reduced number of SVs (Spiwoks-Becker et al., 2001).

Compensation phenomena might also be determined by pantophysin or as yet unidentified isoforms. Moreover, the structural similarity and the widespread tissue

distribution of synaptogyrins and SCAMPs raise the possibility that these molecules substitute for the lack of SypI. Indeed, although the SypI/synaptogyrin I double knock-out mice do not show deficits in the basal glutamate release, they exhibit defects in several forms of synaptic plasticity (Janz et al., 1999). Since these abnormalities were not detected in single SypI knock-out mice, it is likely that SypI and synaptogyrin I display an at least partial functional redundancy.

Interestingly, when neurons from SypI knock-out and wild-type mice were co-cultured to form heterogenotypic networks *in vitro*, neurons lacking SypI were impaired in the ability to form both hetero- and homo-synapses, suggesting that SypI plays a regulatory role in an activity-dependent process of synapse formation (Tarsa and Goda, 2002). Thus, when synaptogenesis occurs under competitive conditions, such as for example in Xenopus neuronal cultures in which only half of the cells had the SypI levels manipulated (Adler et al., 1992b; Adler et al., 1995), an important functional role of SypI in synaptic physiology becomes apparent.

1.6.5 Synaptophysin I regulates the formation of the SNARE complex via interaction with VAMP2

The interaction between v-SNARE VAMP2 and the plasma membrane associated t-SNAREs, syntaxin 1A and SNAP25 underlies the formation of a fusogenic complex, named the SNARE complex, whose function in neuroexocytosis has been elucidated (Gundelfinger et al., 2003). The assembly of the SNARE complex is a spontaneous and irreversible process, and thus must be tightly regulated during the steps of the SV cycle which precede fusion. Moreover, since the t-SNAREs syntaxin 1A and SNAP25 are not confined to the active zones, the assembly of the fusion complex must be not only temporally, but also spatially regulated within the nerve terminal. Indeed, the ability of syntaxin 1A to enter the SNARE complex depends on either the synergistic or

competitive association with the interacting factors Munc 13 and Munc 18, respectively. Analogously, SNAP25 availability to participate in the SNARE complex is regulated by the endosome-associated hepatocyte responsive serum phosphoprotein (Hrs) (Sudhof, 2004).

Syp forms hetero-oligomers with VAMP-2 on the SV membrane that are mutually exclusive with the SNARE complexes (Edelmann et al., 1995). Thus, VAMP2 availability and/or stability has been proposed to depend on SypI, which could play the role of delivering monomeric VAMP2 to the other SNAREs in the right place and at the right time. Indeed, by following the association/dissociation of the SypI/VAMP2 complex in live hippocampal neurons, it was demonstrated that SypI dissociates from VAMP2 prior to fusion of SVs with the plasma membrane. When exocytosis is stimulated by low concentrations of the secretagogue α-Ltx, dissociation of the SypI/VAMP2 complex occurs irrespective of the exocytotic event in one of the steps that precede fusion and make SVs competent for Ca^{2+}-dependent exocytosis (Pennuto et al., 2002). Interestingly, chemical denervation by chronic blockade of postsynaptic receptors results in a reduction of SypI-VAMP2 complexes which is paralleled by an enhancement of neurotransmitter release (Bacci et al., 2001). Moreover, the SypI-VAMP2 complex is absent in both cultured neurons until the onset of synaptogenesis and undifferentiated PC12 cells (Becher et al., 1999a and b). A possible interpretation of these data is that the down-regulation of the SypI-VAMP2 complex observed before synaptogenesis results in high levels of basal synaptic activity typically associated with developing neurons (Matteoli et al., 1992). After synaptogenesis, a developmentally regulated, as yet unidentified, signal which promotes the assembly of the SypI-VAMP2 complex prevents the formation of the SNARE complex causing the decrease of spontaneous synaptic transmission. However, if the timing of the association/dissociation of the Syp/VAMP2 complex is, at least partially, understood, it

remains to be investigated which are the specific signals and/or post-translational modifications that promote dissociation of the complex. Interestingly, the nematode Syp ortholog lacks sites for both known and putative post-translational modifications. Thus, the post-translational control of SypI-VAMP2 complex dynamics might be acquired late in the evolution to meet demand for higher efficiency in the control of synaptic activity.

1.6.6 Does Synaptophysin I participate in the assembly of the fusion pore during neurotransmitter release?

Fusion of a vesicle with the target membrane occurs via the formation of a fusion pore, a transient structure that joins the two apposing membranes before their complete merging (Monck and Fernandez, 1994). Although at present the molecular make-up of this structure is unknown, it is widely accepted to be at least partially proteinaceous, since characteristics such as high speed and versatility to open, flicker, and close, which depend on the kind of stimulus eliciting secretion, cannot be recapitulated by a simple lipid structural model. Based on its propensity to form ion channels upon reconstitution into lipid bilayers, SypI has been proposed to be part of the fusion pore, although its putative presynaptic partner has not been identified yet (Thomas et al., 1988). Recently, these data have been reinforced by the finding that antibodies against SypI are able to alter both the gating and conductance of ion channels from reconstituted bovine neurohypophysial secretory granules (Yin et al., 2002). In addition, channels displaying similar conductance and opening time to SypI particles have been discovered in other systems, such as SVs isolated from Torpedo electric organ (Rahamimoff et al., 1988).

The idea that SypI might participate in the formation of the fusion pore is further strengthened by the high structural homology between SypI and connexins, the gap junction components (reviewed by Simon and Goodenough, 1998). Indeed, despite the low homology in their primary structures (only 13-15%), SypI shares several features

with the connexins: the similar size and membrane topology, a high conservation of the transmembrane domains in both protein families, the ability to form hexamers, and a similar channel conductance. Moreover, a similar amino acid composition is found in the transmembrane domain three of connexin 32, which lines the gap junction pore, and both transmembrane domain three of SypI and transmembrane domain two of ligand-gated channel proteins (Betz, 1990).

Interestingly, when a fusion pore connects a secretory granule with the plasma membrane, a net flux of lipids occurs toward the organelle, causing the dissociation of the structure in its subunits (Monck et al., 1990). It has been reported that in live hippocampal neurons dissociation of Syp homo-oligomers parallels the complete fusion of SVs with the plasma membrane, in agreement with the idea that Syp might be a component of the fusion pore (Pennuto et al., 2002).

1.6.7 Does Synaptophysin I participate in endocytosis?

An involvement of SypI in SV recycling has been proposed on the basis of its interactions with dynamin, cholesterol and AP-1 (Thiele et al., 2000; Daly et al., 2000; Horikawa et al., 2002). The ability of SypI to generate membrane microdomains enriched in cholesterol and SV proteins and its cycling between a homo-oligomeric form in the highly curved SV membrane and a monomeric form when the SV membrane is flattened by collapse into the presynaptic membrane (Pennuto et al., 2002) suggest an important role of the protein in the initiation of the sequential steps that lead to SV fission and retrieval from the donor membrane. Moreover, a noncanonical signal for endocytosis has been identified in the carboxy-terminal tail of SypI (Linstedt and Kelly, 1991a).

The functional importance of the SypI/dynamin interaction has been supported by physiological experiments in which a fusion protein containing the dynamin-binding

domain of SypI was injected into nerve terminals. The injection resulted in SV depletion which was attributed to an impairment of SV recycling and was accompanied by an accumulation of clathrin-coated vesicles near the sites of vesicle fission. These results might imply a role of SypI in both clathrin-independent and clathrin-dependent mechanisms of endocytosis (Daly et al., 2000; Daly and Ziff, 2002). Thus, it is possible to envisage a sequence of events in which SypI/VAMP2 hetero-oligomers dissociate and VAMP2 is recruited to the SNARE complex to promote fusion. SypI might participate in the formation of the fusion pore, and subsequently recruit dynamin to the SV membrane domain, to be released upon GTP binding, thus allowing dynamin assembly and membrane fission.

1.6.8 Synaptophysin and SV biogenesis

A still unresolved issue is the targeting of SypI observed upon expression in epithelial cells (Leube et al., 1989 and 1994). In contrast with other reports (see Cameron et al., 1991), in this heterotypic context SypI was sorted away from endosomal proteins into a special type of vesicles, morphologically indistinguishable from SLMV of neuroendocrine cells.

A reduction in the number of SV has been reported after examination of the retinal rod photoreceptors from SypI knock-out mice, in which synaptoporin is not expressed. This effect was accentuated in periods of synaptic activity, and was accompanied by an increase in SV size and persistence of large vacuoles, maybe owing to the reduced rate of SV reformation (Spiworks-Beker et al., 2001). These results provided the first *in vivo* evidence for the importance of SypI in SV recycling and formation. Two possible mechanisms underlying the role of SypI in SV biogenesis/reformation might be either the SypI-mediated regulation of the endocytotic machinery through its interaction with dynamin I (Daly et al., 2000) or its ability to interact with cholesterol (Thiele et al.,

2000) and various SV proteins (See above). Indeed, through its cholesterol-binding ability, SypI might be involved in the formation of microdomains of specific lipidic composition. In addition, the polytopic structure of the Syp molecule and its ability to form oligomers might promote membrane curvature, facilitating vesicle budding from the donor membrane during biogenesis. Indeed, cholesterol depletion affects the biogenesis of synaptic-like microvesicles in neuroendocrine cells (Thiele et al., 2000; Wang et al., 2000).

1.7 THE SYNAPSINS AS REGULATORS OF SYNAPSE DEVELOPMENT AND FUNCTION

The synapsins are an evolutionarily conserved multigene family of neuron-specific phosphoproteins specifically associated with the cytoplasmic surface of the SV membrane. Three synapsin genes are present in mammals, *synI*, *synII* and *synIII*, located in chromosome X, 3 and 22, respectively. Alternative splicing of each synapsin gene generates distinct isoforms, termed synapsins a, b and b-like (Figure 1.10). All synapsins share a large conserved amino-terminal region, the primary structures of all isoforms diverging in the carboxy-terminal tail.

Since synapsin I is able to interact *in vitro* with various cytoskeletal proteins, such as actin, it has been proposed that the simultaneous interaction of synapsin I with both actin and the SV has the relevant physiological role to link SVs to the actin-based cytoskeleton, maintaining vesicles associated in clusters to form the reserve pool in the vicinity of the active zone (Benfenati *et al.*, 1992). Indeed, video-microscopy experiments demonstrated that the synapsins are both necessary and sufficient for SVs to bind actin filaments (Ceccaldi *et al.*, 1995). Perturbation of synapsin function in a variety of preparations leads to a selective disruption of the reserve pool of SVs and to an increase in synaptic depression, suggesting that the synapsin-dependent clustering of vesicles is required to sustain neurotransmitter release in response to high levels of neuronal activity. More recent studies indicate that, in addition to cause a selective alteration of the reserve pool, perturbation of synapsin activity leads to an inhibition and slowing of the kinetics of neurotransmitter release, indicating a second role for synapsin downstream of vesicle docking to the active zone. Moreover, the role of synapsin in synapse formation and stabilization is well-documented.

1.7.1 Structural organization of the synapsins

Three common regions are found among vertebrate and invertebrate synapsins (Figure 1.10A). At the amino-terminus, all synapsins share a stretch of homology within domain A encompassing residues which represent a consensus phosphorylation site for cAMP-dependent protein kinase (PKA) and Ca^{2+}/calmodulin-dependent protein kinase I (See below), suggesting that phosphorylation of this site is likely to be a relevant, evolutionary conserved regulatory feature (Kao et al., 1999).

The most extensive homology is found within domain C, a large central region of about 300 amino acids. The elucidation of the domain C crystal structure suggests the possibility that synapsin might perform enzymatic roles. Indeed, more than 80% of the Cα atoms of synapsin I domain C are superimposable with those from a group of ATP-using enzymes (Esser et al., 1998). In addition, recombinant domain C regions from all the three synapsin isoforms are able to bind ATP *in vitro* with high affinity, although with striking differences in the Ca^{2+}-mediated regulation of the binding (Hosaka and Sudhof, 1998).

Although derived from three distinct genes by alternative splicing mechanisms, the vertebrate a-type isoforms share a carboxy-terminal region termed domain E. This domain is conserved in selected invertebrate synapsins.

The regions between domains A and C (domain B) and between domain C and the carboxy terminal domain (domains D, G, H, J in the different isoforms) are poorly conserved among the synapsins. However, certain features within these domains are maintained. Of particular relevance is the presence of consensus sites for mitogen-activated protein (MAP) kinase, as well as Ca^{2+}-calmodulin-dependent protein kinase (CaMK) II (See below).

The conservation of a general domain structure and of phosphorylation sites among vertebrate and invertebrate synapsins supports the notion that the basic mechanisms by which the synapsins regulate synaptic transmission have been evolutionary conserved (Kao et al., 1999).

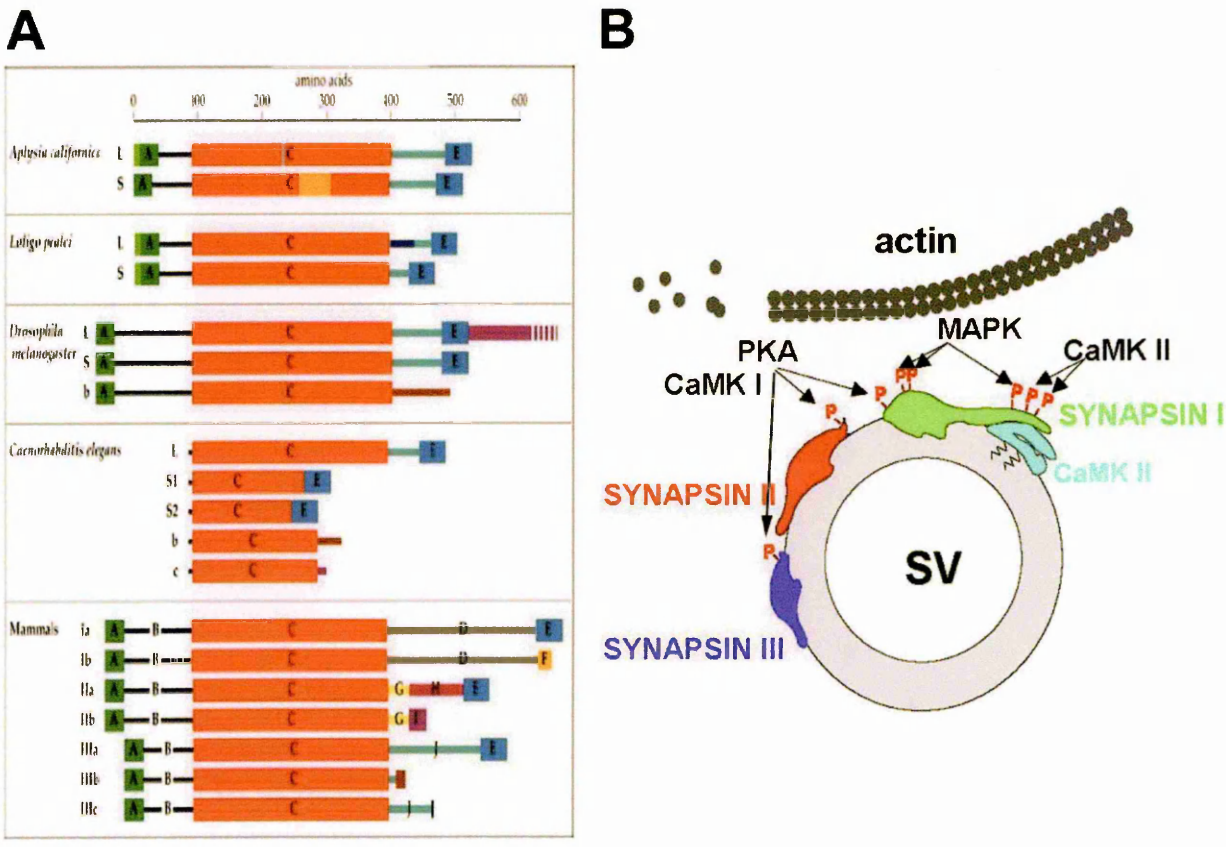

Figure 1.10. Structure and regulation of the synapsin isoforms.
(A) Molecular evolution of the synapsin familty. Synapsins possess multiple domains, which have evolved at different rates throughout the evolution. In both invertebrate and vertebrate synapsins the most conserved domains are A, C and E (modified from Kao et al.,1999). (B) The three synapsin isoforms are shown associated with the SV membrane. Synapsin I is targeted to the vesicle through the interaction with SV-associated CaMKII. Multiple kinases phosphorylate synapsins at distinct sites. Synapsins bind to actin thus tethering SV to the actin cytoskeleton, and facilitate F-actin assembly.

1.7.2 Regulation of synapsin by phosphorylation

Phosphorylation modulates synapsin activity during neuronal transmission (Figure 1.10B). The phosphorylation state of the synapsins is increased under all conditions that promote Ca^{2+}-dependent neurotransmitter release. All synapsins currently identified contain a consensus site for phosphorylation by PKA and by CaMKI (site1) within domain A. Phosphorylation at site 1 leads to subtle changes in the overall conformation of the synapsin molecule (Benfenati et al., 1990), moderately decreases the affinity with which synapsins bind to actin (Bähler and Greengard, 1987), but does not significantly change the binding of synapsin to lipids (Benfenati et al., 1989).

All synapsin isoforms contain three distinct consensus sites for MAP kinase (sites 4, 5 and 6). Sites 4 and 5 are located within domain B, while site 6 is located within domain D and also subjected to phosphorylation by cyclin-dependent kinase (cdk) 5. Similar to the PKA/CaMKI site, phosphorylation at sites 4-6 results in a moderate decrease in the ability of synapsins to interact with actin, but has no effect on the binding of synapsins to SVs (Jovanovic et al., 1996).

In addition, synapsin I, but not synapsin II, is a physiological substrate for CaMKII at two sites located within domain D (sites 2 and 3). Similar consensus sites are present in synapsin III, although their accessibility for phosphorylation remains to be determined. Importantly, phosphorylation at sites 2 and 3 leads to a major conformational change of the synapsin I molecule (Benfenati et al., 1990), as well as to a drastic decrease in the ability of synapsin I to interact with actin (Valtorta et al., 1992) and SVs (Schiebler et al., 1986).

Phosphorylation of synapsin I at sites 1,2 and 3 occurs rapidly after the Ca^{2+} influx that follows the arrival of the depolarizing stimulus and results in dissociation of the

molecule from SVs, which are supposed to become available for exocytosis (Sihra et al., 1989; Torri Tarelli et a., 1992).

1.7.3 Evidence for a role in the control of neurotransmitter release

In order to perturb synapsin function at the nerve terminal level and define its functional role in neuroexocytosis, two main experimental approaches have been used: (i) injection of exogenous synapsin, antibodies to synapsin or peptides derived from evolutionarily conserved synapsin sequences into presynaptic terminals of invertebrate neurons; (ii) genetic ablation of one or more synapsin genes in mice.

Intraterminal injection of synapsin I into the squid giant synapse has been shown to reduce synaptic transmission, whereas the phosphorylated or heat-inactivated forms of the protein are without effect. Introduction of an active form of CaMKII produces opposite effects, increasing the rate of rise and amplitude and decreasing the latency of the postsynaptic potential (Llinas et al., 1985 and 1991). Quantal analysis in this system reveals that synapsin I consistently reduces the number of quantal units available for release, although the probability of release remains unchanged. Fluctuation analysis of the synaptic noise confirmed that injection of dephosphorylated synapsin I reduces the rate of spontaneous and evoked quantal release (Lin et al., 1990).

The results obtained on the squid giant axon have been confirmed in the vertebrate central nervous system on the goldfish Mauthner neurons, where presynaptic injection of synapsin I reduces both spontaneous and evoked synaptic transmission (Hackett et al., 1990). Similar results have been obtained introducing by a freeze-thaw technique synapsin I into rat brain synaptosomes, and measuring the depolarization-induced release of glutamate and noradrenaline (Nichols et al., 1992). Altogether, these results support the model that dephosphorylated synapsin I

provides an inhibitory constraint on neurosecretion that is relieved upon phosphorylation by CaMKII (Greengard et al., 1993).

Another line of evidence pointing to a role of synapsin I on the modulation of neurotransmitter release comes from studies on the physical distribution of the protein in response to trains of action potentials conducted in frog nerve muscle preparations, which show that synapsin I dissociation from the SV membrane is not a prerequisite for fusion and that under high frequency electrical stimulation synapsin I partially dissociates from SV during exocytosis and reassociates with the SV membrane following endocytosis (Torri Tarelli et al., 1990 and 1992). In agreement with these results, phosphorylation of synapsin I in rat brain synaptosomes treated with depolarising agents is associated with a rapid translocation of the protein from the membrane fraction to the synaptosol (Sihra et al., 1989). These data have been recently confirmed in living hippocampal neurons, in which synapsin was found to disperse in the presynaptic terminal and preterminal axon during depolarization and to recluster at SV sites following return to the resting state (Chi et al., 2001). In these studies it was also found that the rates of dispersion and reclustering are indeed controlled by synapsin phosphorylation and dephosphorylation, respectively, and that CaMK-mediated phosphorylation controls SV mobilization at low frequency of stimulation, whereas MAP kinase phosphorylation is recruited at both low and high frequencies of stimulation (Chi et al., 2001 and 2003).

Knock-out mice for either synapsin I, II, I/II, III or I/II/III have been generated (Rosahl et al., 1993 and 1995; Li et al., 1995; Chin et al., 1995; Takei et al., 1995; Ryan et al., 1996; Terada et al., 1999; Feng et al., 2002; Gitler et al., 2004) and show no overt alterations in neuroanatomy and behaviour, indicating that the protein is not essential for viability nor for basic neuronal function. However, despite the absence of gross defects in brain morphology or behaviour, synapsin I and synapsin II (but

not synapsin III) knock-out mice as well as double synapsin I/II and triple synapsin I/II/III knock-out mice exhibited early onset spontaneous and sensory stimuli-evoked (audiogenic) epileptic seizures. Attacks consisted of partial, secondarily generalized "grand mal" attacks followed by post-seizure grooming (Roshal et al., 1995). Electroencephalogram analysis showed that subconvulsive electrical stimulation in the amygdala was able to induce seizures when applied to syn mutant mice (Li et al., 1995). Typically, seizures develop after 2 months of age and mice become more susceptible with age. The incidence of seizures is higher in synapsin II than in synapsin I knock-out mice and is proportional to the number of inactivated synapsin genes. While the synapsin II and I/II knock-out mice have been reported to have impaired contextual conditioning (Silva et al., 1996) and triple knock-out mice exhibited impaired motor coordination and defective spatial learning (Gitler et al., 2004), a detailed analysis of the behavioural phenotype of the synapsin knock-out mice is still lacking.

Ultrastructural examination of the presynaptic terminals of the hippocampal CA3 field of synapsin I knock-out mice reveals that the size of terminals is significantly smaller and the number of SVs in a region >150 nm from the active zone significantly reduced, suggesting a role for synapsin I in the organization of the presynaptic cytoarchitecture (Takei et al., 1995b). Both the number of SVs exocytosed during brief action potential trains and the total recycling vesicle pool are significantly reduced in hippocampal cell cultures derived from synapsin I knock-out mice, while the kinetics of endocytosis and SV repriming appear normal (Ryan et al., 1996).

The selective loss of a major pool of SVs, distal to the active zones, in synapsin I knock-out mice is consistent with the results obtained from the injection of anti-synapsin antibodies in lamprey reticuolspinal axons, which causes the disappearance

of SVs in the reserve pool, while SVs docked at active zones are unaffected. Synapsin depletion is associated with a markedly enhanced depression of neurotransmitter release following high- but not low-frequency stimulation (Pieribone et al., 1995). In addition, quantal analysis of excitatory postsynaptic potentials combined with the ultrastructural study of neuromuscular junctions in synapsin II knock-out mice shows that the lack of synapsin II results in a 40% decrease in the density of SVs in the reserve pool, while the number of docked vesicles remains unchanged. Interestingly, at reduced Ca^{2+} concentrations, quantal release and facilitation of synchronous release are significantly increased in synapsin II knock-out synapses (Samigullin et al., 2004).

The causal link between synapsin deficiency and the epileptic phenotype observed is still far from being elucidated. It has been hypothesized that synaptic depression during repetitive stimulation may contribute to seizure development by causing an imbalance between excitatory and inhibitory systems. Indeed, inhibitory GABAergic interneurons experience high frequency firing that may make GABA release particularly sensitive to the depletion of reserve SV induced by synapsin deletion. Terada and coworkers (1999) investigated in detail the impairment of inhibitory transmission in synapsin I knock-out mice and demonstrated that, in cultured hippocampal synapses from mutant mice, inhibitory, but not excitatory, synapses become easily fatigued and recover slowly from depression upon repeated application of hypertonic sucrose. Stimulated terminals showed a decrease in the number of SV in the reserve pool, but not in readily releasable pool, that was slightly more intense in GABAergic terminals than in glutamatergic ones. However, since hippocampal neurons at developmental stages preceding the onset of synapse formation where used in this study, it remains to be established whether the observed effects derive, at least in part, from a defect in synaptogenesis rather than a change in the mature exocytosis machinery.

Interestingly, a nonsense mutation in the synapsin I gene has been recently identified in a family affected by X-linked epilepsy and mental retardation (Garcia et al., 2004). The consequences of this mutations on synapsin I function remain to be determined.

Notwithstanding the absence of an overt or latent epileptic phenotype, KO mice for the most recently identified member of the synapsin family, synapsin III, also showed an impairment of GABAergic transmission, while excitatory transmission was unaffected. These results leave open the possibility that the function of synapsin III in inhibitory terminals may differ from that at excitatory synapses. A recent study shows that the synapsins have a critical role in maintaining the balance between excitatory and inhibitory synapses in brain networks (Gitler et al., 2004). Excitatory and inhibitory synaptic transmission was differentially altered in synapsin I/II/III triple KO mice: excitatory synapses exhibited normal basal transmission, but decreased number of SV in the reserve pool and marked depression, whereas inhibitory synapses exhibited impaired basal transmission, mild changes in the number of SV and no changes in depression.

1.7.4 Synapsins and short-term plasticity

The study of synapsin-deficient mice suggests specific roles for synapsins in certain forms of synaptic plasticity. Synapsin I-deficient mice exhibit an increase in paired-pulse facilitation (PPF) (Rosahl et al., 1993) but no effect was observed on post-tetanic potentation (PTP) (Rosahl et a., 1995). At variance, synapsin II and I/II KO mice showed no changes in PPF, but a dramatic decrease of PTP (Roshal et al., 1995). These data suggest that synapsin I, but not synapsin II, may function to selectively limit the increase in neurotransmitter release elicited after an initial stimulus. In cholinergic synapses of Aplysia californica, synapsin neutralization by antibody injection produced a virtual disappearance of PTP that was substituted by an intense post-tetanic depression. In the same study, basal synaptic transmission was not altered, but PPF was significantly decreased at physiological Ca^{2+} concentrations. However, decreasing release probability by lowering the Ca^{2+}/Mg^{2+} ratio to remove synaptic depression revealed that PPF was not affected by synapsin neutralization (Humeau et al., 2001). Finally, presynaptic injection of the peptide fragment of domain E in squid giant synapses dramatically decreased postsynaptic potential in response to a single action potential but did not affect PPF (Hilfiker et al., 1998). Thus, most of the available data indicate that PPF is not a primary target of the synapsin action in excitatory terminals, although the function of synapsin on short-term plasticity of inhibitory synapses remains completely unexplored.

1.7.5 Synapsins exert a post-docking control of SV exocytosis

Several results suggest an additional role for synapsins in modulating the release of individual vesicles from the immediately releasable pool. Studies of neurotransmitter release using FM dyes in hippocampal neurons derived form synapsin I-deficient mice showed a decrease in the size of both the total and the immediately releasable pools of vesicles (Ryan et al., 1996). While the former observation was in agreement with the decrease in the reserve pool of SV (Li et al., 1995; Takei et al., 1995), the latter result was rather unexpected, since the readily releasable pool of SV appears relatively intact in synapsin I KO mice. This finding offers the possibility to reinterpret the puzzling increase in PPF in hippocampal synapses in the absence of effects on synaptic depression found in synapsin I KO mice (Rosahl et al., 1993 and 1995): while the increase in PPF could reflect a decrease of initial release probability, the lack of effect of synapsin I deletion on the rate of synaptic depression during repetitive stimulation could be attributable to a simultaneous decrease in release probability and in the size of the functional SV pool.

Direct experimental evidence in support of a post-docking role of synapsins was obtained by the analysis of neuroexocytosis following the presynaptic injection of a peptide corresponding to a conserved region of domain E into the squid giant synapse (Hilfiker et al., 1998). Injection of the domain E peptide was found to inhibit neurotransmitter release in the absence of appreciable changes in the number of docked SV, and to slow the onset and decay of excitatory postsynaptic currents. Slowing of the release kinetics is a highly unusual event and does not simply result from a reduction in neurotransmitter release (See Hilfiker et al., 1999 for a discussion). Thus, it seems likely that the domain E peptide directly affects the kinetics of SV fusion events. However, the physiological role of synapsin domain E is currently unknown.

A post-docking action of synapsins is likely to be involved also in the decrease of evoked IPSCs (eIPSCs) observed in CA3 pyramidal neurons from hippocampal slices of P10-14 synapsin I KO mice (Terada et al., 1999). Mutant mice showed a decrease in the amplitude and an increase in the coefficient of variation of eIPSCs, while the amplitude of miniature IPSCs was not affected, suggesting that synapsin I deficiency reduces the efficiency of inhibitory synaptic transmission by decreasing the number of SV released by a single action potential

Altogether these results suggest that the synapsins are also involved in the post-docking steps of release, by directly or indirectly regulating priming and/or fusion reactions thus playing a role in determining the rate and the number of docked SV released in response to an action potential. This post-docking action could be accounted for by interactions of the synapsins with the dynamic actin cytoskeleton present at the active and periactive zones and/or with presynaptic proteins involved in the priming/fusion steps.

On the one hand, it has been recently shown that the synapsin domain E peptide has the ability to inhibit the binding of endogenous synapsins to actin, an effect that is shared by other synapsin peptides (i.e. peptides encompassing regions of the conserved C domain) that also alter the kinetics of release (Hilfiker et al., 2005), suggesting that an interaction with actin at the active zone may play a role in the post-docking effects of synapsin. On the other hand, synapsins have been recently shown to interact with the SV-associated G protein Rab3A and to modulate Rab3 cycling and GTPase activity in nerve terminals (Giovedì et al., 2004a,b). As Rab 3A has been proposed to limit the amount of neurotransmitter released in response to the Ca^{2+} signal in a late step that follows docking and priming (Geppert et al., 1997), the post-docking effect of synapsins can be achieved by the removal of the Rab3-mediated inhibitory constraint on quantal release.

1.7.6 Synapsins regulate neuronal development and synaptogenesis

During the formation of the nervous system, the appearance of synapsin I has been found to correlate with synaptogenesis in various animal species (De Camilli et al., 1983; Mason, 1986). In hippocampal neurons developing in culture before the establishment of synaptic contacts synapsin I immunoreactivity is distributed in the distal axons and growth cones and becomes readily clustered at newly formed synapses. Interestingly, most of the synapsin I immunoreactivity is associated with SV precursors even in the absence of synaptic contacts (Fletcher et al., 1991). The study of axonal transport of synapsin I points to the existence of three pools of the protein (Baitinger and Willard, 1987). One pool leaves the cell body immediately after synthesis, while the other two pools, which account for more than 90% of the axonally transported synapsin I, are released from the cell body with a delay of more than one day. The first and the second pools are delivered via fast axonal transport, travelling at the speed of membrane-associated proteins and SV markers, whereas the third pool travels slowly, at the speed typical of cytoskeletal proteins. Moreover, it appears that changes in the phosphorylation of synapsin I occur during its transport via the slow pathway (Petrucci et al., 1991), suggesting that compartment-specific phosphorylation of synapsin I, affecting the molecular interactions with the vesicles and the cytoskeleton, may be implicated in determining a correct intracellular traffic of SVs.

The observation that the peak of synapsin I and II expression closely matches the onset of synaptogenesis has suggested that the proteins may play a role in this process. Overexpression of synapsin IIb in a neuroblastoma x glioma hybrid cell line affects cell differentiation, with an increase in the number of varicosities and in the number of SVs per varicosity. In addition, the effect is accompanied by an increase in cell-cell contacts with the characteristics of synaptic specializations (Han et al., 1991). The expression of individual synapsin isoforms in fibroblasts produces an evident reorganization of the

actin cytoskeleton, which results in the appearance of elongated processes containing large bundles of actin (Han and Greengard, 1994).

Whether synapsin I and II exert overlapping functions during neuronal development is unclear. Ablation of synapsin II with antisense oligonucleotides in cultured hippocampal neurons impairs the elongation of both the axon and dendrites and causes a selective reduction in the content of several SV proteins (Ferreira et al, 1994). If synapsin II expression is suppressed after axonal elongation but prior to synapse formation, neurons fail to develop synapses, while if antisense treatment follows synapse formation, most synaptic contacts are lost (Ferreira et al., 1995). Thus, synapsin II may participate both in neurite extension and establishment and maintenance of synaptic contacts.

Similarly to synapsin II, the genetic ablation of synapsin I results in a delay in both axonogenesis and synaptogenesis in cultured hippocampal neurons derived from synapsin I knock-out mice (Chin et al., 1995). A further examination of hippocampal neurons in culture derived from either single or double knock-out mice for synapsin I and II indicate that the two isoforms participate to different processes during neuronal development. Synapsin I-deficient neurons display an impaired synaptogeneis, while synapsin II-deficient neurons exhibit an alteration in neurite elongation. Surprisingly, both effects are partially restored by deletion of both synapsins (Ferreira et al., 1998).

A role for the synapsins in neurite extension has been confirmed by the *in vivo* study of axon elongation in Xenopus embryonic spinal neurons in which the phosphorylation state of synapsins was manipulated. Expression of a mutated form of synapsin IIa devoid of the PKA site or introduction of antibodies directed against this site decreased neurite outgrowth, while expression of a synapsin IIa version bearing a mutation which mimics constitutive phosphorylation at the PKA site increases neurite outgrowth (Kao et al., 2002).

Purified synapsin I loaded into Xenopus embryonic spinal neurons promotes the functional maturation of the neuromuscular junctions formed *in vitro* by the injected neurons with muscle cells. At these developing synapses, both spontaneous and evoked postsynaptic currents display increased amplitude and frequency, with a precocious appearance of a more mature form of spontaneous neurotransmitter release characterized by a bell-shaped distribution of the current amplitudes (Lu et al., 1992). Untrastructural analysis of neuromuscular junctions formed by synapsin I-loaded neurons are characterized by the presence of a higher number of SVs clustered at precociously organized acive-zone structures (Valtorta et al., 1995). Similarly, recombinant synapsin IIa injected into Xenopus embryos promotes the functional maturation of neuromuscular synapses formed *in vitro* by the injected neurons (Schaeffer et al., 1994).

At variance with synapsin I and II, synapsin III is expressed early during neuronal development and its expression is downregulated in mature neurons (Ferreira et al., 2000). Mice lacking synapsin III exhibited a marked delay in neurite outgrowth, no change in SV density, an increase in the size of the recycling pool of SV and a significant decrease in synaptic depression (Feng et al., 2002), in sharp contrast with what observed in synapsins I and II KO mice (Li et al., 1995; Ryan et al., 1996). These data indicate a unique non redundant role for synapsin III in the regulation of neuronal development.

AIM OF THE WORK

The main goal of this project is to address the molecular mechanisms underlying the establishment of a functional neurosecretory apparatus during neuronal development, whit a particular focus on synaptic vesicles (SVs), the organelles devoted to the storage and release of neurotransmitters.

Even before they form a fully functional synapse, the pre- and postsynaptic cells contain many of the components necessary for synaptic transmission. However, before they are capable of sustained and effective neurotransmission, both the pre- and postsynaptic partners must undergo a process of maturation. Assembly of a functional synapse is a process which is globally orchestrated. It has recently been proposed to involve transport along the axon of "packets" containing most/all of the proteins destined to the synapse (Almenar-Queralt and Goldstein, 2001). However, contrasting data have also been presented, which indicate a separate sorting for some of the exocytic proteins and the formation of mature SVs exclusively after the establishment of a synaptic contact (Ahmari et al., 2000 and Zhai et al., 2001).

My aim is to identify the processes involved in the establishment of a pool of mature SVs, ready to fulfil the demand of rapid and efficient stimulus-secretion coupling needed at synapses. To this purpose, I primarily investigate two aspects of the SV life cycle at different stages of neuronal differentiation.

First, I study how SVs become endowed with the highly specialized complement of proteins which make these vesicles competent for efficient neurotransmitter release. Several of these components have been characterized and linked to at least putative functions in the regulation of the SV life cycle. However, little is known about how proteins are targeted specifically to SVs. The situation is complicated by the fact that no

feature that makes a protein recognizable as a SV protein has been identified thus far. In the absence of specific targeting signals, the delivery of proteins to SVs is likely to rely on a complex network of protein-protein and protein-lipid interactions, as well as post-translational modifications.

Almost all the present knowledge about the intracellular traffic routes taken by membrane proteins destined to SVs during the biogenesis of these organelles derives from data collected studying the biogenesis of synaptic-like microvesicles (SLMVs) of neuroendocrine cells. In particular, the trafficking of synaptophysin I (SypI) an abundant four-transmembrane domain protein of both SVs and SLMVs has been extensively analyzed: SypI leaves the TGN in constitutive secretory vesicles, appears at the surface, undergoes cycles of endo-exocytosis possibly involving an endosomal compartment and is eventually targeted to SLMVs. However, it remains to be investigated to what extent these results can be extrapolated to the targeting of SV components and whether other newly synthesized SLMV membrane proteins take the same intracellular membrane trafficking route to their final destination.

In this study I address the targeting of a critical component of the SV, the v-SNARE VAMP2, which interacts with plasma membrane t-SNAREs to form the SNARE complex that drives SV fusion. Therefore, the presence of VAMP2 on SVs determines their function, as demonstrated by the ablation of fast Ca^{2+}-triggered SV exocytosis in the neurons of VAMP2 knock-out mice (Schoch et al., 2001; Deak et al., 2004). VAMP2 function may be regulated by SypI. The two proteins interact with each other on the SV membrane, and VAMP2 cannot participate in the SNARE complex until it dissociates from SypI, which suggests that the assembly of the SypI-VAMP2 complex restricts the availability of SVs for fusion (Edelmann et al., 1995; Pennuto et al., 2002).

I employ fluorescence resonance energy transfer analysis coupled to high resolution videomicroscopy to monitor the interaction between SypI and VAMP2 during the exo-

endocytotic cycle in living mature neurons in the attempt to expand previous data obtained in the laboratory (Pennuto et al., 2002). Live imaging of fluorescent chimeras of various SV proteins, including VAMP2 and SypI, fused to spectrally separated variants of GFP, expressed in both cultured hippocampal neurons and non-neuronal cells enables me to explore the molecular events which govern the targeting of VAMP2 on SVs and, more generally, the delivery of SV components on the vesicles.

The second aspect of the functional maturation of SVs addressed in this study concerns the molecular events involved in the regulation of SV dynamics at early stages of neuronal differentiation. Neurotransmitter secretion in the developing nervous system may participate in axonal pathfinding and navigation by modulating the rate and direction of axonal growth. Indeed, in developing neurons the assembly of a functional apparatus for neurotransmitter secretion does not require a contact with the postsynaptic target (Zakharenko et al., 1999), although at this stage the rudimentary machinery for quantal neurotransmitter secretion is distributed throughout the whole axonal surface. Maturation of this machinery during synaptic formation would improve the fidelity of synaptic transmission.

While in mature neurons neurotransmitter secretion depends on the exocytosis of well-characterized neurotransmitter-containing synaptic vesicles, the identity of the organelles involved in neurotransmitter release along growing processes is debated. Furthermore, although neurotransmitter release from isolated axons appears to be Ca^{2+}-dependent, it is unclear whether release is from vesicular stores or directly from the cytosol. In addition, it has been suggested that the SV recycling pathway in developing neurons is ontogenetically related to the constitutive endosomal membrane recycling pathway found both in neuronal and non-neuronal cells (Matteoli et al., 1992).

This study is aimed at answering the question as to whether and how SV recycling is regulated in developing neurons. As a model system we exploit the axonal growth cones

associated with hippocampal neurons during early stages of differentiation in culture. Indeed, the neuronal growth cones are particularly suitable to study the dynamics of membrane carriers in vivo since they offer the advantage of large compartments already endowed with much of the machinery for neurotransmitter release, which is known to occur both spontaneously and upon stimulation (Matteoli et al., 2004). However, the study of membrane trafficking in neuronal growth cones has been hampered by the high molecular and morphological heterogeneity of the intracellular membrane organelles that they contain. Thus, although a number of morphological and functional criteria have been employed to overcome these issues, the molecular profiles and functional roles of the organelles present in the growth cones remain to be established.

Here, I study SV dynamics in growth cones by videomicroscopy imaging of fluorescent chimeras of SV proteins fused to different coloured variants of GFP expressed using HIV-derived lentiviral vectors (Naldini et al., 1996; Lotti et al., 2002). These viral vectors are particularly suitable for efficient and stable transduction of non-dividing cells, such as neurons, without compromising their viability and developmental program. The use of endocytic tracers allows to determine the recycling properties of SVs in the growth cones.

During the process of synaptic maturation, SVs become concentrated at the growth cone, where they appear to be excluded from the distal, actin-rich portion. In addition, at mature synapses a population of SVs appears to be constrained by their link to the actin-based cytoskeleton. Thus, it appears likely that SV-associated proteins play a role in regulating their mobility in developing and mature nerve terminals. The best candidates for mediating these effects are the synapsins, a family of phosphoproteins specifically associated with the cytosolic side of the SV membrane, whose phosphorylation state is regulated by various kinases and phosphatases on distinct sites (De Camilli et al., 1990; Greengard et al., 1993). The synapsins, which are able to bind SVs to actin in a

phosphorylation-dependent manner, are likely candidates for mediating the differential mobility and distribution of the vesicle pools in the growth cone. This hypothesis is tested by analyzing the distribution and dynamics of the vesicle pools in the growth cones of hippocampal neurons in which the expression and the state of phosphorylation of the synapsins are manipulated.

MATERIALS AND METHODS

2.1 MATERIALS

The monoclonal antibody against synaptic vesicle protein 2 (SV2) was provided by Dr. K. Buckley (Harvard University, Boston, MA, U.S.A.). Monoclonal antibodies against Synaptophysin I (SypI), VAMP2 and Synaptotagmin I (cytosplasmic domain, Syt_C and lumenal domain, Syt_L) and the polyclonal antibodies against VAMP1 and Syntaxin 13 were from Synaptic Systems (Göttingen, Germany). The polyclonal antibody against SypI has been previously described (Valtorta *et al.*, 1988). The monoclonal antibodies against microtubule associated protein 2 (MAP2) and the hemagglutinin protein of human influenza virus were from Roche Diagnostics, (Indianapolis, IN, U.S.A.). The 3E6 monoclonal antibody against green fluorescent protein (GFP) was from Quantum Biotechnologies (Montreal, Canada). The anti-synapsin I/II monoclonal antibody (clone 19.11), the anti-sinapsin I polyclonal antibody G177 and the phosphorylation state-specific anti-synapsin polyclonal antibodies, G257 (phosphosite-1) and RU19 (phosphosite-3), were prepared and characterized at The Rockefeller University (New York, NY, U.S.A.) (Valtorta et al., 1988; Menegon et al., 2000). The phospho-synapsin (Ser9) antibody (phosphosite-1-specific) used in western blotting was from Cell Signaling Technology (Beverly, MA, U.S.A.). The anti-ERK antibody (K-23) was from Santa Cruz (Santa Cruz, CA, U.S.A.). The monoclonal antibody against early endosome antigen 1 (EEA1) was from BD Biosciences (San Jose, CA, U.S.A.). The monoclonal anti-human transferrin receptor antibody was from Zymed (San Francisco, CA, U.S.A.). The monoclonal anti-giantin antibody was developed by Dr. MA. De Matteis (Consorzio Mario Negri Sud, Chieti, Italy). The H4A3 anti-human Lamp-1 monoclonal antibody was obtained from the Developmental Studies Hybridoma Bank, University of

Iowa, Department of Biological Sciences, Iowa City, IA, U.S.A.). TRITC, FITC and AMCA-conjugated secondary antibodies and pure rabbit IgG antibodies were from Jackson ImmunoResearch (West Grove, PA, U.S.A.). TRITC-conjugated Phalloidin was from Sigma-Aldrich (St. Lois, MO, U.S.A.). Peroxidase-conjugated anti-rabbit was from Bio-Rad (Hercules, CA, U.S.A.). The enhanced chemiluminescence detection system was from Amersham Bioscience (Little Chalfont Buchinghamshire, UK) and BCA protein assay reagent was from Pierce (Rockford, IL, U.S.A.). Cytochalasin D, H89, Forskolin were from Calbiochem (La Jolla, CA, U.S.A.). N^6,2'-O-dibutyryladenosine 3':5'-cyclic monophosphate (BT-cAMP) and Filipin III were from Sigma-Aldrich. FM 4-64 and FITC-conjugated transferrin wre from Molecular Probes (Eugene, OR, U.S.A.). Taipoxin was purchased from Venom Supplies (Tanunda, South Australia). α-latrotoxin was a kind gift of Dr. Alexander Petrenko (New York University, New York, U.S.A.).

All chemicals were diluted in KRH (in mM: 150 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2 $CaCl_2$, 10 glucose, and 10 HEPES/Na, pH 7.4) at the indicated concentrations.

2.2 DNA CONSTRUCTS

pECFP-N3 and pEYFP-N3

The ECFP (*enhanced cyan fluorescent protein*) and EYFP (*enhanced yellow fluorescent protein*) cDNAs were amplified from the vectors pECFP-N1 and pEYFP-N1 (Clontech, Palo Alto, CA) by PCR with the following oligonucleotides: forward, 5'-GGGGGGATCCATCGCCACCATGG-3' and reverse, 5'-GGGGGTGTACAGCTCGTCCATGCC-3'. BamHI and BsrGI restriction sites, introduced with the forward and reverse primers respectively, are underlined. The PCR products were cleaved with BamHI and BsrGI and cloned into the pEGFP-N3 vector

(Clontech), from which the cDNA fragment encoding the EGFP was removed by digestion with the same enzymes, to generate the pECFP-N3 and pEYFP-N3 vectors. All the PCR products were sequenced prior to cloning into expression vectors.

pSypI-ECFP, pSypI-EYFP, SypIΔC-ECFP

Both the SypI full length cDNA (921 bp), inserted into the pBlueScript vector (Stratagene, La Jolla CA, USA), and the deletion mutant SypIΔC (702 bp), lacking the last 73 amino acids of the protein, cloned into the pEGFP-N3 vector were a kind gift of Dr. R. Leube (Johannes Gutenberg University Mainz, Mainz, Germany). SypI cDNA was amplified from the pBlueScript vector by PCR with the following oligonucleotides: forward, 5'-GGGGGAAGCTTCAGCAGCAATGGACGTG-3' and reverse, 5'-GGGGGGATCCGCTGCTGTAGTAGCAGTAGGTCTTGGGCTCCAC CCCTTCATCTGATTGGAGAAGGAGGTGG-3'. HindIII and BamHI restriction sites, introduced with the forward and reverse primers respectively, are underlined. In addition, the reverse oligonucleotide introduced a linker of 13 amino acids between SypI and the fluorescent proteins, and whose amino acidic sequence is KGVEPKTYCYYSS (Grote *et al.*, 1995). The amino acidic sequence was converted to the nucleotidic sequence, optimised to the most prevalent "codon usage" of mammalian cells. After digestion with HindIII and BamHI restriction enzymes the PCR product was inserted into the HindIII/BamHI cleaved multicloning site of the vectors pECFP-N3 and pEYFP-N3, to generate the vectors pSypI-ECFP and pSypI-EYFP, respectively.

EGFP from the pSypIΔC-EGFP vector was substituted by ECFP extracted from the pECFP-N3 vector by a NheI/BsrGI cut, thus generating the pSypIΔC-ECFP vector.

pECFP-VAMP2 and pEYFP-VAMP2

VAMP 2 full length cDNA (351 bp) cloned into pBlueScript, was a kind gift of Drs. C. Montecucco and O. Rossetto (University of Padova, Italy). The VAMP2 cDNA was amplified by PCR from the pBlueScript vector with the following oligonucleotides: forward, 5'-GGGGTGTACAAGATGTCGGCTACCGCTGCCAC-3' and reverse, 5'-GGGGGCGGCCGCTTAAGTGCTGAAGTAAAC-3'. BsrGI and NotI restriction sites, introduced with the forward and reverse primers respectively, are underlined. the PCR product s cleaved with BsrGI and NotI restriction enzymes was inserted into the BrsGI/NotI cleaved vectors pECFP-N3 and pEYFP-N3, to generate the pECFP-VAMP 2 and pEYFP-VAMP 2 vectors.

pVAMP2-GFP and pVAMP2-EYFP

The VAMP2-GFP expressing vector was a kind gift of Dr. R. Scheller (Stanford University School of Medicine, Stanford, CA, U.S.A.).

The VAMP2 cDNA was amplified by PCR from the pECFP-VAMP2 vector with the following oligonucleotides: forward, 5'-GGGGCTCGAGATGTCGGCTACCGCTGCC-3' and reverse, 5'-GGGGAAGCTTAGTGCTGAAGTAAACGATGATG-3'. XhoI and HindIII restriction sites, introduced with the forward and reverse primers respectively, are underlined. After digestion with XhoI and HindIII restriction enzymes the PCR product was inserted into the XhoI/HindIII cleaved vector pEYFP-N1 to generate the pVAMP2-EYFP vector.

pECFP-VAMP1

Rat VAMP1 full-length cDNA (357 base pairs) cloned into the pBlue-Script vector (Stratagene, La Jolla, CA, U.S.A.) was obtained from Drs. C. Montecucco and O.

Rossetto (University of Padua, Italy). VAMP1 cDNA was amplified by PCR with the following oligonucleotides: forward, 5'GGGGTGTACAAGATGTCTGCTCCAGCTCAGCC-3'; and reverse, 5'-GGGGGCGGCCGCTCAAGTAAAAATGTAGATTA-3'. BsrGI and NotI restriction sites, introduced by the forward and reverse primers, respectively, are underlined. The resultant BsrGI/NotI PCR fragment was cloned in frame at the carboxy-terminal of ECFP in the corresponding sites of pECFP-N3 vector (Clontech), generating the pECFP-VAMP1 vector.

pECFP-VAMP1 and pEYFP-VAMP2

The ECFP-VAMP2/CtVAMP1 and EYFP-VAMP1/CtVAMP2 expressing vectors were produced by swapping the amino-terminal domains of VAMP1 (aa 1-99) and VAMP2 (aa 1-96) extracted by a BsrGI/BclI cut from pECFP-VAMP1 and pEYFP-VAMP2, respectively.

pEYFP-TfR

Human transferrin receptor (hTfR) cDNA (2300 base pairs) cloned into the pCMV5 plasmid was a gift of Dr. D. Zacchetti (San Raffaele Scientific Institute, Milan, Italy). TfR cDNA was extracted by an EcoRI cut and cloned in frame at the carbxy-terminal of EGFP in the corresponding site of pEGFP-C3 vector (Clontech). EGFP was substituted by EYFP extracted from pEYFP-N3 vector by a NheI/BsrGI cut, thus generating the pEYFP-TfR vector.

pSyntaxin13-ECFP

Syntaxin13 cDNA cloned in frame at the C-terminal of EGFP in pEGFP-N3 was a kind gift of Dr. V. Faundez (Emory University, Atlanta, GA, U.S.A.). EGFP was substituted

by ECFP extracted from pECFP-N3 vector by a BamHI/BsrGI cut, thus generating the pSyntaxin13-ECFP vector.

pECFP-Rab11 and pECFP-Rab5

The pEGFP-Rab11 and pEGFP-Rab5vector were kind gifts of Drs. M. Zerial (Max Planck Institute, Dresden, Germany) and C. Bucci (Università degli Studi, Lecce, Italy), respectively. EGFP was substituted by ECFP extracted from pECFP-N3 vector by a NheI/BsrGI cut, thus generating the pECFP-Rab11 and pECFP-Rab5 vectors.

pHA-Dyn and pHA-Dyn K44A

The pHA-Dyn and pHA-Dyn K44A vectors in which HA-tagged dynamin (Dyn) and its mutant Dyn K44A are cloned into pcDNA3 were developed by Dr. S.L. Schmid (Scripp Research Institute, La Jolla, CA, U.S.A.) (Damke et al., 1994).

pRRL-SypI-EYFP(pgk) and pRRL-ECFP-VAMP2(pgk)

The fragment SypI-EYFP was amplified by PCR from the plasmid pSypI-EYFP using the following oligonucleotides: forward, 5'-GGGGACCGGTCAGCAGCAATGGACGTGGTG-3' and reverse, 5'-GGGGGTCGACTTACTTGTACAGCTCGTCCATG-3'. After AgeI/SalI digestion the SypI-EYFP insert was cloned into the self-inactivating lentiviral vector RRL.ppt.hPGK.GFP.pre.sin-18 (Lotti et al., 2002), in which EGFP was removed by an AgeI/SalI cut, to produce pRRL-SypI-EYFP(pgk). The RRL.ppt.hPGK.GFP.pre.sin-18 plasmid contains a central polypurine tract (ppt) to enhance transduction in non-dividing cells, the woodchuck hepatitis virus posttranscriptional regulatory element to increase expression of the targeted transgene, and a deletion in the U3 region of the long terminal repeat. Inserts are placed under the control of an internal, constitutive phosphoglycerol

kinase (PGK) promoter. The fragment ECFP-VAMP2 was extracted from pECFP-VAMP2 (Pennuto et al., 2002) by a NheI/NotI cut and cloned into pBS SK II (Stratagene) digested with EcoRV after filling of the NheI and NotI sites to generate pBS SK ECFP-VAMP2. ECFP-VAMP2 extracted from pBS SK ECFP-VAMP2 by a XmaI/SalI cut was ligated into RRL.ppt.hPGK.GFP.pre.sin-18 digested with AgeI/SalI to generate pRRL-ECFP-VAMP2(pgk).

pRRL-ECFP-Syn Ia and pRRL-ECFP-Syn Ia S9A

The cDNAs of rat synapsin Ia and the S9A mutant cloned into the plasmid pEGFP-C1 (Clontech) were provided by Dr. H-T Kao (Nathan Kline Institute for Psychiatric Research, Orangeburg, NY, U.S.A.). EGFP was substituted with ECFP extracted from ECFP-C1 by a NheI/BsrGI cut and ligation to produce the plasmids pECFP-Syn Ia and ECFP-Syn Ia S9A. ECFP-Synapsin Ia and ECFP-Synapsin Ia S9A were extracted from the respective plasmids by an AgeI/SalI digestion and cloned into the RRL.ppt.hPGK.GFP.pre.sin-18 plasmid to generate pRRL-ECFP-Syn Ia(pgk) and pRRL-ECFP-Syn Ia S9A(pgk). The two digested fragments were also introduced into the plasmid pRRLsinPTT.CMV.GFPpre (Lotti et al., 2002) in which EGFP was removed by an AgeI/SalI cut to produce the pRRL-ECFP-Syn Ia(CMV) and pRRL-ECFP-Syn Ia S9A(CMV) plasmids, in which the inserts are placed under the control of the cytomegalovirus (CMV) promoter.

2.3 VIRUS PRODUCTION AND TRANSDUCTION OF NEURONS

Lentiviral stocks pseudotyped with the vesicular stomatitis G protein (VSV-G) were prepared by transient cotransfection of 293T cells using the Ca^{2+}-phosphate precipitation method with the transfer vector, the pCMVΔR8.74 plasmid encoding Gag, Pol, Tat, and Rev, and the pMD.G plasmid encoding VSV-G. The culture medium

containing viral particles was harvested at 48 h and 72 h after transfection, and viral preparations were concentrated by ultracentrifugation to increase titer. Viral titers (infectious particles) were determined by transduction of 293T cells with serial dilution of the viral stocks and evaluation of transduction efficiency by flow cytometry scoring of fluorescent cells.

Four hours after plating of neurons, coverslips were placed in a clean dish containing glia-conditioned medium (MEM supplemented with 1% N2 supplement (Invitrogen), 2 mM glutamine (Biowhittaker), 0.1% ovalbumin, 1 mM sodium pyruvate (Sigma-Aldrich), and 4 mM glucose) and incubated for 10-15 h at 37° C in a 5% CO_2 humidified atmosphere in the presence of viral supernatant at 1-10 multiplicity of infection. After transduction, neurons were returned to the original dishes and maintained in culture in glia-conditioned medium.

2.4 CELL CULTURES

Hela cells

Hela cells were grown on either plastic dishes or glass coverslips at 37°C in a 5% CO_2 humidified atmosphere in Dulbecco's modified Eagle's medium (Biowhittaker, Verviers, Belgium), supplemented with 10% fetal calf serum (Hyclone, Logan UT, USA), 1% L-glutamine and 100 U/ml penicillin/streptomycin (Biowhittaker).

Embryonic hippocampal neurons

Primary neuronal cultures were prepared from the hippocampi of E18 embryos from either Sprague-Dawley rats (Charles River Italica, Calco, Italy), synapsin I knock-out mice (Li et al., 1995), synapsin II knock-out mice, or synapsin I/II/III triple knock-out mice (Feng et al., 2002) as previously described (Banker and Cowan, 1977). Synapsin knock-out mice were genotyped as described in Li et al. (1995) and Feng et al. (2002).

Mice were maintained in a specific pathogen free animal facility and all experiments were performed in strict accord with experimental procedures approved by the San Raffaele Scientific Institute animal care and use committee and the Italian National Ministry of Health.

Briefly, hippocampi were dissociated in 0.25% (wt/vol) trypsin and cells were plated at a density of 10000-20000/cm^2 on poly-L-lysine-coated glass coverslips in minimal essential medium (MEM) (Life Technologies, Paisley, Scotland), supplemented with 10% horse serum (Hyclone) and 3.3 mM glucose. After 3-4 hrs to allow the cells to adhere, the coverslips were transferred upside down over a monolayer of cortical astroglial cells (Booher and Sensenbrenner, 1972), grown in MEM supplemented with 1% N2 supplement (Life Technologies), 2 mM glutamine, 4 mM glucose, 1 mM sodium pyruvate and 1 mg/ml ovalbumin.

Cortical astroglial cells

Cells were prepared from 1-2 day post-natal rats. The hemispheres were dissociated in 0.25% trypsin and plated on plastic dishes at 37°C in a 5% CO_2 humidified atmosphere in MEM, supplemented with 10% horse serum, 3.3 mM glucose, 20 U/ml penicillin/streptomycin and 2 mM glutamine.

24 hrs before the addition of hippocampal neurons plated on coverslips, the cells were fed with MEM supplemented with 1% N2 supplement, 2 mM glutamine, 4 mM glucose, 1 mM sodium pyruvate and 1 mg/ml ovalbumin.

2.5 TRANSFECTION METHODS

Transfection of hippocampal neurons

Neurons were transfected at 3 days *in vitro* (DIV) using 25-kDa polyethylenimine (PEI 25) (Sigma-Aldrich). PEI (28 nmoles/dish) and plasmid DNA (2.5 μg/dish) were

diluted in 50 µl of 150 mM NaCl in separate tubes, mixed and vortexed four times within 12 min. Immediately before transfection, coverslips were placed in a clean 35 mm Petri dish, rinsed with minimal essential medium supplemented with 10% horse serum, 2 mM glutamine, and 3.3 mM glucose and then incubated for 2 h at 37° C in a 5% CO_2 humidified atmosphere with 1 ml of the same medium containing the PEI 25/DNA mixture. After incubation the coverslips were returned to the original dishes and maintained in culture until 15 DIV. Living transfected neurons were imaged at room temperature in Krebs-Ringer's solution (KRH) (150 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES/Na, pH 7.4). For the experiments in which changes in the localization of the fluorescent proteins upon exocytosis were monitored (Figure 3.2), transfected neurons at 15 DIV were rapidly rinsed with KRH supplemented with 2 mM EGTA (KRH/EGTA) and subsequently incubated for 40 min at 37° C in 5% CO_2 in the same solution containing 0.1 nM α-latrotoxin (α-Ltx) (a gift of Dr. A. Petrenko, New York University Medical Center, NY, U.S.A.).

Transfection of Hela cells

For immunofluorescence studies, Hela cells grown on glass coverslips were transfected with 1.5-3 µg of DNA using a standard Ca^{2+}-phosphate precipitation protocol. A solution of 250 mM $CaCl_2$ containing DNA was added dropwise to an equal volume of HEPES buffered saline solution 2X (HeBS) (280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4, pH 7.05). After 20 min of incubation, the precipitates were added to the cells. 16 hrs later, cells were rinsed twice with Hank's Balanced Salt solution (HBSS) (Biowhittaker) and incubated with fresh medium. Cells were analysed 48 hrs later. For biochemical studies, Hela cells grown on 6 cm plastic dish were transfected with

Lipofectamine2000 (Stratagene, La Jolla CA, USA) according to the manufacturer's instructions using 4 μg of each plasmid and were analysed 24 hrs later.

2.6 CELL LABELLING PROTOCOLS

Cells to be processed for immunofluorescence were fixed for 30 min with 4% paraformaldehyde, 4% sucrose in 120 mM sodium phosphate buffer (pH 7.4), rinsed with phosphate-buffered saline (PBS) and incubated overnight at 4°C with the primary antibody appropriately diluted in goat serum dilution buffer (GSDB) (15% goat serum, 450 mM NaCl, 0.3% Triton X-100, 20 mM sodium phosphate buffer, pH 7.4). Incubation with the appropriate secondary antibodies (Jackson ImmunoResearch) was carried out for 1-2 h at room temperature. Specimens were then washed three times within 30 min with high salt buffer (500 mM NaCl, 20 mM sodium phosphate buffer, pH 7.4) and once with 5 mM sodium phosphate buffer, pH 7.4. Coverslips were mounted with 70% glycerol in PBS supplemented with phenylenediamine (1 mg/ml; Sigma-Aldrich) as an anti-bleaching agent.

TRITC-conjugated phalloidin was added during incubation of the secondary antibodies when indicated. For detection of synapsin phosphorylation, the Cy3-conjugated phosphosite-3-specific anti-synapsin I antibody (RU19) (Menegon et al., 2002) was added after the standard double labelling protocol. Incubation with the conjugated antibody was performed in the presence of 10 μg/ml rabbit IgG and was preceded by a 20 min blocking reaction in the same solution. For the Syt_L-based exo-endocytic assay, neurons were incubated for 15 min with the Syt_L antibody diluted in KRH, rapidly washed with KRH/EGTA, fixed, detergent-permeabilized and counterstained with the second primary antibody followed by TRITC and FITC-conjugated secondary antibodies.

For FM 4-64 uptake experiments in growth cones, neurons were incubated with FM 4-64 (10 μM) diluted in KRH for 1 min at room temperature, washed 3 times by complete medium substitution with KRH during 1 min, fixed and processed for immunofluorescence.

For FM 4-64 uptake and release at synapses, the dye (10 μM) was loaded into recycling SVs of 15 DIV hippocampal neurons using a depolarizing solution containing KRH supplemented with 45 mM KCl. The incubation was carried out for 60 s at room temperature, and was followed by rinsing for 15 min with a 2 ml/min flow of KRH containing 10 μM CNQX (Tocris, Ellisville, MO) and 1 μM TTX (Tocris, Ellisville, MO). After the washing protocol, FM4-64 staining was imaged using a 530-595 nm bandpass filter for excitation and a 615 nm longpass filter for emission and a 63X oil immersion objective. After incubation for 30 min at 37°C in the same solution in the absence or presence of Tpx, cells were imaged to measure the FM4-64 content of either intoxicated or untreated synapses. Corresponding differential interference contrast (DIC) images were used to identify the swollen boutons in the Tpx-treated neurons. The intensity of FM4-64 fluorescence at single synapses was measured before and after treatment.

For cell surface detection of VAMP2-GFP, living hippocampal neurons were incubated for 10 min at 37° C with anti-GFP antibody diluted in 10% horse serum, 2 mM glutamine, and 3.3 mM glucose, rinsed briefly in PBS and fixed as described above. Cells were incubated for 1 h at room temperature with TRITC-conjugated anti-mouse antibody (Jackson ImmunoResearch) in GSDB, washed and mounted as described above. For cell surface detection of fluorescent chimeras in Hela cells, fixed specimens were incubated with anti-GFP antibody, which also recognizes the other spectral variants CFP and YFP, diluted in GSDB devoid of detergent and processed as described above. Afterwards, cells were incubated with Texas red-conjugated anti-mouse antibody

(Jackson ImmunoResearch) in GSDB devoid of detergent, washed and mounted as described above.

2.7 BIOCHEMICAL METHODS

Western blotting

Cells were solubilised by scraping with solubilisation buffer (1% SDS, 2 mM EDTA, 10 mM HEPES-Na, pH 7.4) and immediately frozen in liquid nitrogen. Lysates were then boiled for 3 min and sonicated. Equal amounts of proteins were subjected to SDS-polyacrylamide gel electrophoresis (Laemmli, 1970), and transferred to nitrocellulose as previously described (Towbin *et al.*, 1979).

Filters were blocked for 1 hr at room temperature (RT) with 5% non-fat dry milk or bovine serum albumin in Tris-buffered saline (TBS) (200 mM NaCl, 50 mM Tris.HCl, pH 7.4), incubated for two hrs with anti GFP, anti Syp or anti VAMP2 antibodies, washed 5 times for 5 min with TBS supplemented with a detergent (either 0.1% Triton X-100 or 0.1% Tween 20), incubated for 1 hr at RT with the secondary antibody (peroxydase-conjugated secondary anti-mouse or anti-rabbit antibody, 1:10000), washed 5 times for 5 min with TBS supplemented with the same detergent and finally developed by chemiluminescence with the ECL system (Amersham, Little Chafont, UK).

Cross-linking analysis and Immunoprecipitation

In order to detect the formation of heterocomplexes between exogenous SypI and VAMP2 expressed in Hela cells, cells were rinsed twice with cold PBS, and harvested by scraping with PBS followed by centrifugation for 3 min at 1000 g. Extraction was performed with 200 µl of extraction buffer containing: KCl 140 mM, EDTA 2 mM, Hepes-NaOH 10 mM, pH 7.4 and 1% (v/v) Triton X-100 for 1 h at 4° C under rotation,

followed by centrifugation for 5 min at 1000 g to eliminate nuclei. The supernatant was further centrifuged for 10 min at 20,000 g to remove cell debris. GammaBind™ G-sepharose beads (Amersham Biosciences, Uppsala, Sweden) were washed three times in extraction buffer and incubated with 150 µl of extraction buffer in the presence of 2.6 µg of anti-HA monoclonal antibody for 2 h at 4° C under rotation. A volume of total cell extract containing 400 µg of proteins was then added to the beads and incubated overnight at 4° C under rotation in the presence of protease inhibitors (Sigma-Aldrich). The beads were pelletted for 1 min at 200 g and washed three times in extraction buffer. Finally, the beads were resuspended in sample buffer, boiled for 7 min, centrifuged for 1 min at 14,000 g and analysed on SDS-PAGE followed by western blotting. The supernatants from the immunoprecipitation procedure were analysed in parallel.

In order to analyze the formation of SypI and VAMP2 hetero- and homo-complexes in hippocampal neurons, 15 DIV neurons were rinsed once with KRH/EGTA and subsequently incubated for 45 min at room temperature in the same solution supplemented with 0.5 mM Disuccinimidyl Suberate (DSS) (Pierce, Rockford, Illinois, U.S.A.). At the end of the incubation, TRIS-NaOH (pH 7.4) was added to the final concentration of 100 mM. After 30 min, the neurons were processed for immuno-precipitation with either polyclonal anti-SypI or monoclonal anti-VAMP2 antibodies as described above. In other experiments, SVs purified from rat forebrain through the step of sucrose density gradient (SG2 fraction; Huttner *et al.*, 1983) were subjected to chemical cross-linking with DSS (0.2 mg/ml final concentration) for 1 h at room temperature. The reaction was blocked by the sequential addition of 100 mM glycine and Laemmli stop buffer (Laemmli, 1970) and the samples were subjected to SDS-PAGE and immunoblotting.

Stripping and reprobing.

After exposure to X-ray films, occasionally filters were subjected to stripping and reprobing. To this aim, they were submerged in a large volume of stripping buffer (100 mM β-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mM Tris-HCl, pH 6.7) and incubated at 50° C for 30 min with gentle agitation. They were then washed twice for 10 min with TBS supplemented with detergent (either 0.1% Triton X-100 or 0.1% Tween 20) and incubated for 1 hr at RT with 5% non-fat dry milk or bovine serum albumin in TBS.

After stripping, filters were either quickly washed in TBS, air-dried and stored at 4° C, or directly subjected to a new immunoblot analysis, as described above.

2.8 VIDEOMICROSCOPY AND QUANTIFICATION

Specimens were viewed with a Zeiss (Oberkochen, Germany) Axiovert 135 inverted microscope equipped with epifluorescence optics. Images were recorded with a C4742-98 ORCA II cooled charge-coupled device camera (Hamamatsu Photonics, Hamamatsu City, Japan) and processed using Image Pro Plus 4.5 (Media Cybernetics, Silver Spring, MD) and Adobe Photoshop 6.0 (Adobe System, San Jose, CA, U.S.A.).

For the quantification of synaptically versus extra-synaptically located protein, an average of 450 synaptic boutons belonging to 6 distinct cells from at least two different experiments were analyzed for each condition. Six representative 12 bit (1024×1024 pixels) images of axonal processes were acquired with a 40× oil immersion objective. Acquisition parameters were maintained constant in all experiments. In the case of endogenous VAMP2, FP-VAMP2 coexpressed with cytosolic EYFP and SypI-FP, the staining pattern for endogenous SV2 was used to prepare a binary mask in which each spot corresponded to a synaptic bouton. In all other cases, the synaptic mask was prepared using the distribution pattern of FP-SypI coexpressed in the same cells. Diffusion of the chimeras was expressed as the ratio between the amount of protein

(number of pixels × average fluorescence) localized outside and within synaptic boutons (F_{out}/F_{in}).

For the quantification of the Syt_L antibody internalization, neurons double labeled with Syt_L and Syt_C recognized by TRITC and FITC-conjugated secondary antibodies, respectively, were imaged and the intensities of the TRITC and FITC signals were quantified within the whole growth cone area by generating a binary mask based on the FITC signal (Syt_C). The index of Syt_L internalization [$F(Syt_L)/F(Syt_C)$] expresses the ratio between the TRITC and FITC intensities.

The pseudocolor spectrum surface plot showed in Figure 3.27 was generated using a dedicated command of Image Pro Plus 4.5 in order to obtain a three-dimensional representation of the intensities of the SypI-EYFP fluorescence, with warmer hues corresponding to pixels of higher fluorescence intensity.

For the evaluation of synapse formation, I counted the number of synapses positive for both VAMP2 and either ECFP-SynI or ECFP-SynI S9A in infected neurons, and the number of VAMP2-positive synapses in uninfected neurons in random fields of view. To reduce possible variability due to different levels of expression of the two synapsin chimeras, neurons were stained with saturating concentrations of an anti-GFP antibody which recognizes both exogenous synapsins. Infection efficiency was determined by comparing the number of GFP-positive cell bodies to the total number of VAMP2-positive cell bodies in low magnification random fields. VAMP2 immunoreactivity was measured in an area of 4×4 pixels at the centre of either GFP-positive or negative synapses.

2.9 FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET) MEASUREMENTS BY VIDEO-DIGITAL IMAGING MICROSCOPY

Donor photobleaching video-digital imaging.

Elucidation of the structures and functions of macromolecules, whose characteristic dimensions are < 0.01 μm, requires the use of highly sophisticated techniques, such as FRET. Albeit application of light microscopy is limited by the fact that the optical resolution level imposed by the wavelength of light and by diffraction phenomena is > 0.2 μm, FRET surpasses these limitations and to measure energy transfer from an excited donor to an acceptor fluorophore as a function of their distance. Since the efficiency of the process is inversely related to the sixth power of chromophore separation (3), FRET confines the range of finite energy transfer to distances generally < 0.01 μm, distances that can "separate" only interacting proteins.

The donor photobleaching technique allows to evaluate protein-protein interactions by measuring the nanosecond decay kinetics of the electronic excited-state of chromophores. In fact, the fluorescence lifetime of a chromophore is sensitive to excited-state reactions, such as FRET. The fluorescence lifetime (τ_f) defines the average amount of time that a molecule spends in the excited state (S*) upon absorption of a photon of light. While in the excited state, the molecule is vulnerable to irreversible photodestruction, named photobleaching, at a rate (k_{bl}), that, over time, diminishes the total population of chromophores. The photobleaching process manifests itself as an exponential decay in the fluorescence intensity: the τ_{bl} photobleaching time defines the exposure time required for the fluorescence intensity I to fall to 37% of its initial value I_o. The τ_{bl} values can be fitted to an exponential decay as follows:

$$I = I_o e^{-\tau/\tau bl} \quad (1)$$

$$I = I_o e^{-\tau/\tau} = (^1/_e)\, I_o = 0.37\, I_o$$

The τ_f fluorescence lifetimes range typically from picoseconds to nanoseconds, while the τ_{bl} photobleaching times range from milliseconds to minutes, allowing them to be measured. Thus, one method of determining excited-state lifetimes is to measure the photobleaching kinetics of the donor (Jovin and Arndt-Jovin, 1989). The longer the time that the donor fluorophore spends in the excited state, the greater the probability that it undergoes an irreversible excited-state chemical destruction and the faster the photobleaching. Any process, such as FRET, that shortens the excited-state lifetime of the chromophore, protects it from photobleaching. The τ_{bl} photobleaching time is thus inversely proportional to the excited-state τ_f lifetime. The photobleaching time of the donor, measured in the presence or absence of the acceptor, allows to calculate the FRET efficiency E, that is the measure of the energy transferred from the donor to the acceptor.

Upon excitation by the absorption of light at a rate k_{ex}, deactivation from the excited state can occur via the spontaneous emission of fluorescence with a rate k_f, or via "dark" mechanisms such as internal conversion (k_{ic}), intersystem crossing to the triplet state (k_{isc}) and FRET (k_{FRET}). Since FRET involves the transfer of energy from an excited donor molecule to the acceptor through dipole-dipole coupling, the resonance conditions necessary for this process dictate that the fluorescence emission spectrum of the donor overlaps with the absorption spectrum of the acceptor molecule (Bastiaens and Squire, 1999). These phenomena can be expressed in a quantitative manner by the following relationships for the rate of energy transfer (k_{FRET}) and the transfer efficiency E (Jovin and Arndt-Jovin, 1989):

$$k_{FRET} = (R_o/R)^6 k_d \quad (2)$$

$$E = [1+(R/R_o)^6]^{-1} \quad (3)$$

$$R_o^6 = k^2 Jn^{-4} Q_o(8.8 \times 10^{-25}) \quad (4)$$

R and R_o are respectively the separation distance between donor and acceptor and the critical distance at which the transfer efficiency is 50%, which typically ranges between 2-7 nm. E varies inversely to the sixth power of the distance between the chromophores. n is the refractive index of the intervening medium. Q_o ($\equiv k_f/k_d$), where k_d is the total rate constant for deactivation of the excited state ($= k_f + k_{ic} + k_{isc}$). The fluorescence lifetime τ_f is defined as $1/ k_d$. J is the spectral overlap integral and k^2 is the orientation factor, which defines the geometric relationship between donor and acceptor and that can lie in the range 0-4, but is usually taken to be $^2/_3$, a value valid for a mobile donor and/or acceptor chromophore or for the case of random orientation between the two.

When FRET occurs between donor and acceptor molecules, τ_f decreases to τ'_f, while τ_{bl} increases to τ'_{bl}. Thus, E can be calculated as:

$$E = 1-(\tau'_f/\tau_f) = 1-(\tau_{bl}/\tau'_{bl}) \quad (5)$$

FRET measurements by video-digital imaging microscopy were performed on single synaptic boutons (varicosities), making it possible to limit the analysis of protein present on SVs clustered to the sites of exocytosis.

Photobleaching digital imaging microscopy (pbDIM).

Expression vectors encoding fluorescent proteins were co-transfected at a ratio of 1:4 (donor:acceptor). Cells (15-18 DIV) were washed once with KRH and incubated in the same solution in the presence or absence of 6 nM Tpx for 30 min at 37° C in 5% CO_2; the cells were then washed twice with KRH. Images were acquired within 30-45 min after treatment of the cells. The sample was irradiated at the wavelength of 436 nm +/-10 nm to excite the donor (ECFP chromophore), and photobleaching registered by taking images of the donor at the wave length of 470 nm +/-10 nm at regular intervals, with a CCD camera-equipped microscope as described above. For each sample series of 20 images, acquired with a time exposure of 1500 msec and with a minimum interval between consecutive images, were taken.

Processing of the series of images.

From the first image of the series, a binary mask was prepared, in which each spot, corresponding to a synaptic bouton, was converted to a white signal on a black background. Fluorescent spots which moved quickly along the axon (and which presumably represented traveling packets) were excluded from the analysis. Analysis of the sequences of images was performed with the use of a macro developed by Dr. David Dunlap for Image Pro Plus 4.0 (Dunlap and Valtorta, 2003). For each pixel position with a white mask signal the program measures how the intensity of the fluorescence varies with time, from the first to the last image of the series. The time-series data were fitted pixel-by-pixel to an exponential decay function to obtain a map of the τ_{bl} photobleaching times.

When FRET occurs between donor and acceptor fluorophores, the time constant for donor photobleaching increases (Jovin and Arndt-Jovin, 1989). Thus, the efficiency (E) of FRET was calculated as the percent change in the average time constant of donor

photobleaching measured in specimens transfected with the SV-located acceptor fluorescent proteins (τ_{sv*sv}), with respect to that measured in specimens transfected with cytosolic EYFP acceptor (τ_{sv*cyt}).

$E = 1 - (\tau_{sv*cyt}/\tau_{sv*sv})$.

One of the advantages of this method for measuring FRET is that the measurements do not depend on absolute values of fluorescence. Indeed, we found no significant correlation between initial intensities of fluorescence and photobleaching rates ($R \approx 0.4$). The photobleaching time constants were found to have skewed distributions which became normal after logarithmic transformation. Therefore, data were analyzed using the natural logarithms of the photobleaching time constants, and efficiencies and statistics were derived by re-transformation of the pertinent values.

RESULTS

3.1 STUDY OF SYNAPTIC VESICLE PROTEIN SORTING

3.1.1 Overexpressed VAMP2 is sorted to the axon of hippocampal neurons in culture

A chimera made by enhanced cyan fluorescent protein (ECFP) fused to the cytosolic N-terminal portion of the SV protein VAMP2 (Figure 3.1) was expressed in rat embryonic hippocampal neurons in culture, and its targeting was tracked by videomicroscopy imaging of live cells. Transfected neurons were maintained in culture until they acquired full functional maturation (DIV 15; Valtorta and Leoni, 1999). In the axon, the overexpressed fluorescent chimera showed a diffuse pattern of distribution, similar to that displayed by soluble enhanced yellow fluorescent protein (EYFP) transfected in the same cells (compare Figures 3.2A and A'). However, ECFP-VAMP2 was enriched in puncta which were stained by the endogenous SV markers SV2 and SypI (Bajjalieh *et al.*, 1994; Navone *et al.*, 1986), and could therefore be identified as synaptic boutons (Figures 3.2B-B'' and 3.2C-C''). Only a small fraction of VAMP2-positive puncta were observed to move in either anterograde or retrograde direction (our unpublished results), identifying them as travelling packets (Ahmari *et al.*, 2000; Nakata *et al.*, 1998; Kaether *et al.*, 2000).

In order to assess whether at the level of puncta ECFP-VAMP2 was present in functional SVs, exocytosis was stimulated by α-latrotoxin (α-Ltx) (Figure 3.2D). When applied in the absence of extracellular Ca^{2+}, α-Ltx causes massive SV exocytosis, which is not followed by endocytosis (Valtorta *et al.*, 1988). As previously described (Pennuto *et al.*, 2002), α–Ltx-stimulated exocytosis resulted in the formation of two distinct

populations of synaptic boutons with different size. Exocytosis-induced insertion of the chimera into the axonal plasma membrane generated a ring of fluorescence at the periphery of the large boutons.

Virtually no colocalization of the VAMP2-positive neurites with the somato-dendritic markers microtubule-associated protein 2 (MAP2; Figure 3.2F-F'') and transferrin receptor (TfR; not shown) (Kosik and Finch, 1987; Cameron *et al.*, 1991) was observed, indicating that the chimera is specifically targeted to the axon of transfected cells. Only when the expression levels of ECFP-VAMP2 were exceedingly high the chimeric protein was found also in dendrites (not shown). These cells, which frequently exhibited signs of toxicity, were excluded from all subsequent analyses.

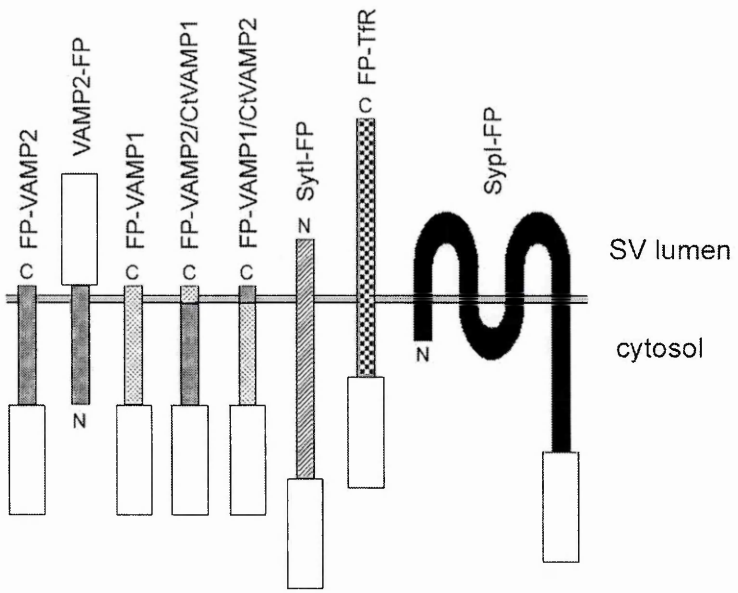

Figure 3.1. Schematic representation of fluorescent chimeras.
The SV proteins VAMP2, VAMP1, SytI and SypI, as well as TfR were fused to either enhanced cyan, yellow, or green fluorescent proteins (FP), here represented by white boxes. ECFP-VAMP2/CtVAMP1 and EYFP-VAMP1/CtVAMP2 chimeras were produced by swapping the N-terminal regions of VAMP1 and VAMP2 fused to ECFP and EYFP, respectively. The orientation of the proteins in the membrane lipid bilayer is indicated.

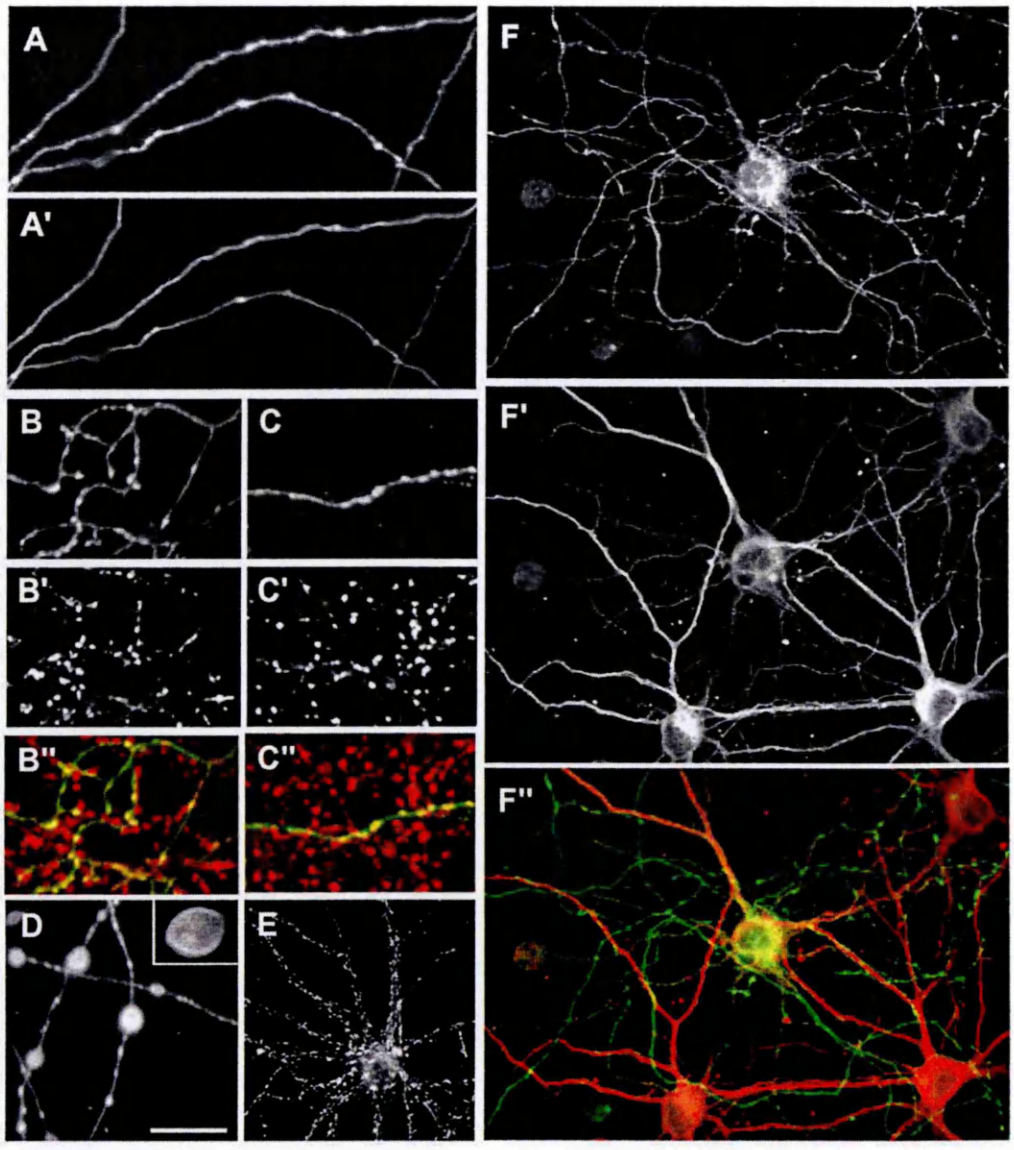

Figure 3.2. Overexpression leads to ECFP-VAMP2 mis-sorting along the axon of transfected hippocampal neurons.

(A and A') 15 DIV hippocampal neurons co-expressing ECFP-VAMP2 (A) and cytosolic EYFP (A'). ECFP-VAMP2 overexpression results in a diffuse distribution of the protein along the axon of the transfected cells, although the protein is enriched in puncta. Some of these puncta show also enhanced staining for soluble EYFP and probably represent sites of increased thickness of the axon. (B-B''; C-C'') ECFP-VAMP2 (B and C) colocalizes in puncta with the endogenous SV markers SV2 (B'), and SypI (C'). (B'' and C'') Merge of previous images. ECFP-VAMP2 is shown in green, SV2 and SypI are in red. (D) Neurons expressing ECFP-VAMP2 treated for 30 min with 0.1 nM α-Ltx. The chimera is present in functional synapses, which undergo swelling because of the massive toxin-stimulated exocytosis. The incorporation of ECFP-VAMP2 into the plasma membrane after exocytosis is apparent after focusing on the surface of a large bouton (inset). No major changes in the appearance of extra-synaptic ECFP-VAMP2 are visible upon α-Ltx stimulation. (E) Distribution of endogenous VAMP2 in hippocampal neurons at 15 DIV. (F-F'') Neurons expressing ECFP-VAMP2 (F) and stained for the somatodendritic marker MAP2 (F'). (F'') Merge of the previous images. ECFP-VAMP2 is shown in green, MAP2 in red. ECFP-VAMP2 distribution is mainly polarized to the axons.

Bar, 10 μm for A-C'', 17 μm for E and F-F'', 6 μm for D, 3 μm for the inset in D.

To determine the proportion of ECFP-VAMP2 that diffused along the axon with respect to the amount that was localized at the level of puncta, the fluorescence intensity and number of VAMP2-positive pixels which colocalized with endogenous SV2 was quantified and compared with the intensity and number of those that did not colocalize with SV2. This analysis indicated that the bulk of ECFP-VAMP2 was located outside synaptic boutons (Figure 3.3). A similar analysis was performed for endogenous VAMP2 revealed by indirect immunofluorescence. In this case, the majority of VAMP2 appeared to be confined to synaptic boutons (Figures 3.2E and 3.3). The lack of diffusion of endogenous VAMP2 in both immature (DIV 3; see below) as well as mature (DIV 15) neurons grown under similar conditions suggests that the diffuse distribution of ECFP-VAMP2 is the result of its overexpression in transfected cells.

To rule out the possibility that the diffusion of ECFP-VAMP2 was due to the presence of the fluorescent protein fused to the cytosolic N-terminal portion of VAMP2, we analyzed the distribution of a chimera in which GFP is fused to the intralumenal C-terminal portion of VAMP2 (Figure 3.1). The VAMP2-GFP chimera, transfected in hippocampal neurons, showed a diffuse pattern of fluorescence similar to that displayed by ECFP-VAMP2 (Figure 3.4A, compare with Figure 3.2A). Quantification of the diffusion of VAMP2-GFP with respect to endogenous SV markers gave results similar to those obtained for ECFP-VAMP2 (Figure 3.3).

3.1.2 Exogenous VAMP2 is present in vesicles which constitutively fuse with the axonal plasma membrane

The possibility that the extrasynaptic VAMP2 chimera was localized on the plasma membrane was tested by applying anti-GFP antibodies to live, unfixed neurons transfected with VAMP2-GFP, whose targeting to the cell surface would have exposed the intralumenal GFP tag. In these cells, the staining pattern determined in the axon by

the anti-GFP antibody was virtually identical to that of VAMP2-GFP, indicating that exogenous VAMP2 is actually exposed to the extracellular surface of the plasma membrane along the axon of transfected cells (Figure 3.4A and A'). As expected, the anti-GFP antibody could not stain the chimera in the Golgi complex (Figure 3.4B and B'). Thus, exogenous VAMP2 was mis-sorted from SVs to membrane carriers which mediate constitutive transport to the plasma membrane.

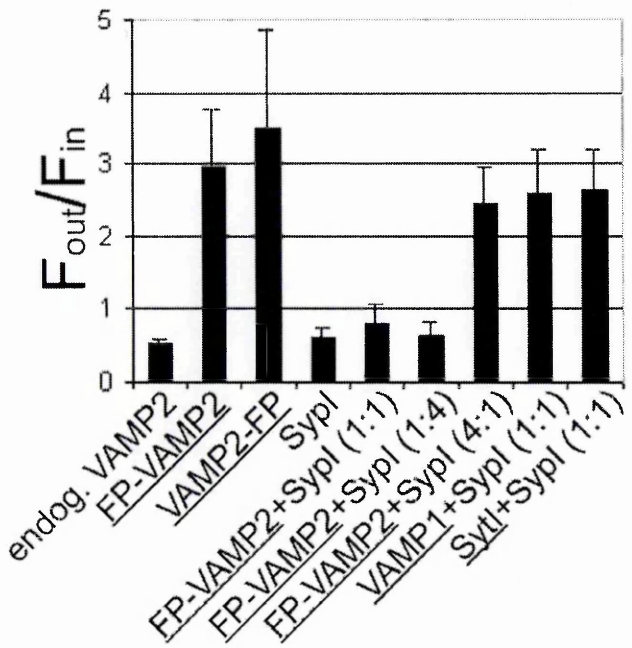

Figure 3.3. Quantification of the distribution of the SV fluorescent proteins.
The ratio (F_{out}/F_{in}) (± S.D.) between the amount of exogenous fluorescent SV proteins present outside and inside synaptic boutons was calculated and compared with the distribution of endogenous VAMP2. For each experimental condition, the protein analyzed is underlined.

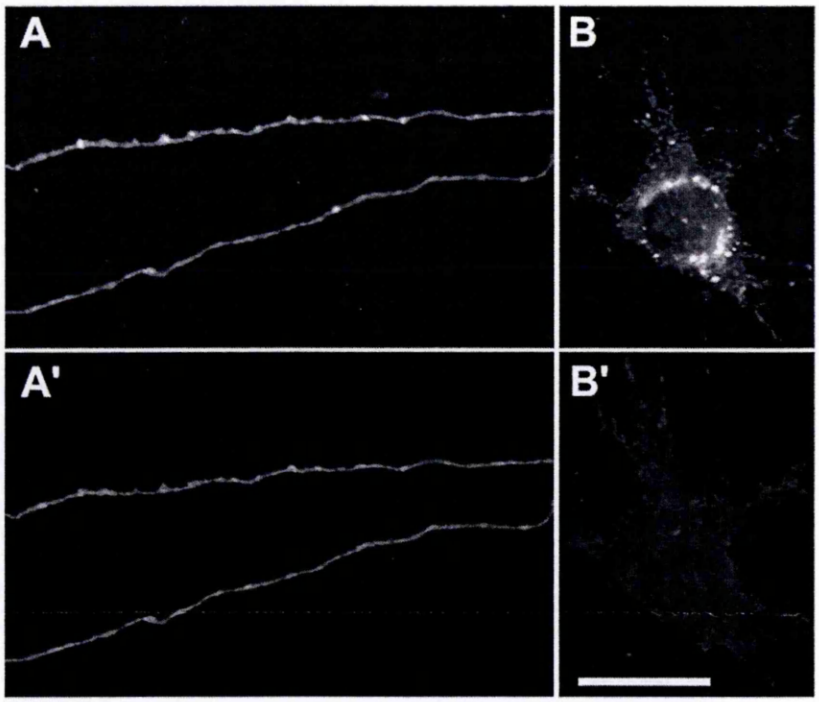

Figure 3.4. Exogenous VAMP2 is present on the axonal plasma membrane.
Hippocampal neurons (15 DIV) expressing the VAMP2-GFP chimera, in which GFP is fused to the intravesicular domain of the protein.
(A) VAMP2-GFP diffuses along the axon of transfected cells. (A') Surface staining of live unfixed neurons with an anti-GFP antibody. (B-B') Cell body from the same transfected neuron. Due to membrane integrity, VAMP2-GFP in the Golgi compartment (B) is not accessible to the anti-GFP antibody (B').
Bar, 10 μm.

3.1.3 Synaptophysin I directs the sorting of VAMP2 to synapses

Since Synaptophysin I (SypI) directly interacts with VAMP2 (Calakos and Scheller, 1994; Washbourne *et al.*, 1995; Edelmann *et al.*, 1995; Galli *et al.*, 1996; Pennuto *et al.* 2002) and has been involved in the process of SV biogenesis (Thiele *et al.*, 2000), we investigated whether the protein might affect VAMP2 sorting in hippocampal neurons. Interestingly, the chimera SypI-EYFP, in which EYFP is fused to the cytosolic C-terminal tail of SypI (Figure 3.1), always appeared to be selectively localized at synaptic boutons, and was never observed to diffuse along the axonal membrane, independently of the level of expression of the protein or the developmental stage of the cells (Figures 3.3 and 3.5). These results are in agreement with previous reports of localization of the chimera into functional SVs (Pennuto *et al.*, 2002).

SypI-EYFP was co-transfected in hippocampal neurons together with ECFP-VAMP2 using various ratios of expression plasmids for the two proteins. When ECFP-VAMP2 and SypI-EYFP were cotransfected in a 1:4 ratio, exogenous VAMP2 showed a well defined punctate distribution, with very low levels of protein diffused outside the puncta. ECFP-VAMP2 positive puncta precisely coincided with SypI-EYFP puncta and with endogenous SV2 (not shown), defining them as synapses. Similar results were obtained when ECFP-VAMP2 and SypI-EYFP expression plasmids were cotransfected in a 1:1 ratio (Figures 3.3 and 3.5).

In contrast, cotransfection of ECFP-VAMP2 and SypI-EYFP in a 4:1 ratio led to a clear diffusion of ECFP-VAMP2 along the axons, a pattern virtually indistinguishable from that observed when the chimera was expressed in the absence of exogenous SypI (Figure 3.5, compare with Figure 3.2A). Indeed, in this case the proportion of diffused VAMP2 was similar to that observed for VAMP2 overexpressed in the absence of SypI (Figure 3.3). The lowest amount of ECFP-VAMP2 plasmid used in the cotransfection experiments corresponded to that used in the previous experiments (i.e. an amount sufficient to lead to diffusion of the chimera along the axons).

In order to assess the specificity of the effect of SypI on VAMP2 sorting, EYFP was fused to the cytosolic C-terminal tail of the single-pass SV protein synaptotagmin I (SytI), to generate the SytI-EYFP chimera (Figure 3.1). When neurons were co-transfected with the expression vectors for ECFP-VAMP2 and SytI-EYFP in a 1:1 ratio, both chimeras displayed a diffuse pattern of distribution along the axons (Figures 3.5), although they were enriched at the level of synaptic sites marked by antibodies to endogenous SV2 (not shown).

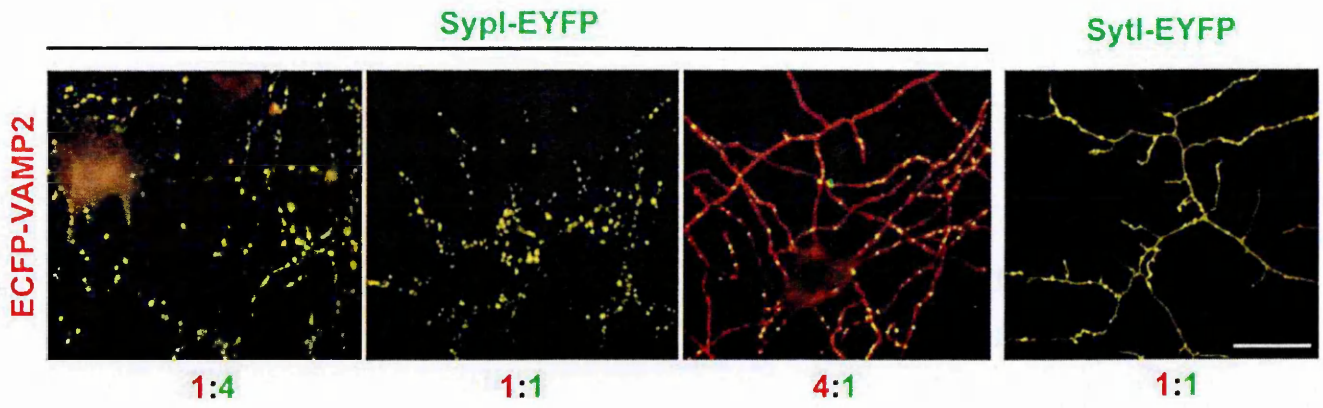

Figure 3.5. SypI corrects the mis-sorting of exogenous VAMP2.
Hippocampal neurons (15 DIV) co-transfected with ECFP-VAMP2 (red) and either SypI-EYFP (left) or SytI-EYFP (right) (green). For each condition the ratio of transfected plamids is shown with the corresponding colour coding.
SypI exerts a dose-dependent rescue of mis-sorted VAMP2 to its correct synaptic localization.
Co-expression of ECFP-VAMP2 and SytI-EYFP results in diffusion of both chimeras along the axons of transfected neurons. Bar, 10 μm.

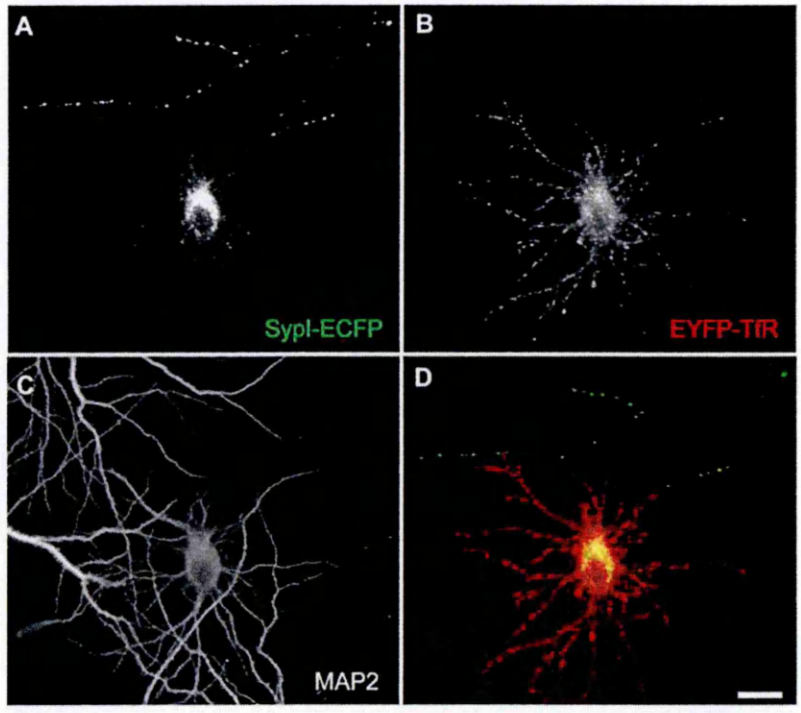

Figure 3.6. Polarized trafficking of TfR to the somatodendritic compartment is not altered by SypI overexpression.
Hippocampal neurons (15 DIV) co-transfected with the expression plasmids for EYFP-TfR and SypI-ECFP in a 1:4 ratio. (A) SypI-ECFP is visible at the level of the Golgi complex and in synaptic boutons along the axon. (B) EYFP-TfR colocalizes with SypI-ECFP in the Golgi complex and is trafficked to the somato-dendritic compartment, labelled by an anti-MAP2 antibody (C). (D) Merge of (A) and (B): SypI-ECFP is shown in green, EYFP-TfR in red.
Bar, 10 μm.

3.1.4 The expression of Synaptophysin I does not affect sorting of proteins destined to the somato-dendritic compartment

The possibility that expression of exogenous SypI might alter polarized membrane trafficking in neurons was tested by analyzing the distribution of the somatodendritic protein Transferrin Receptor (TfR) in neurons overexpressing SypI. The EYFP-TfR chimera, in which the TfR N-terminal end was fused to EYFP (Figure 3.1), was co-expressed in hippocampal neurons together with SypI-ECFP. Neurons were cotransfected with the expression plasmids for EYFP-TfR and SypI-ECFP in a 1:4 ratio. The two chimeras colocalized in the Golgi complex and were then specifically sorted to their correct subcellular compartments. Thus, SypI-ECFP was exclusively present at synaptic sites along the MAP2-negative axon, whereas EYFP-TfR was localized in the MAP2-positive somatodendritic compartment of the transfected neurons (Figure 3.6).

3.1.5 Synaptophysin I does not exert a general effect on SV protein sorting

The dependence of VAMP2 sorting on SypI expression prompted us to investigate whether SypI also regulates the sorting of other SV proteins. In order to explore this possibility, we studied the distribution of SytI-EYFP in hippocampal neurons expressing SypI-ECFP. When SytI-EYFP was overexpressed together with SypI-ECFP in either a 1:1 or a 1:4 ratio, it showed a diffuse pattern of distribution (Figure 3.7A and A', and data not shown), similar to that displayed by SytI-EYFP when overexpressed together with either ECFP-VAMP2 (Figure 3.5) or with soluble ECFP (not shown). The pattern of distribution of SytI-EYFP was reminiscent of that of ECFP-VAMP2 expressed in the absence of SypI (Figures 3.2 and 3.3). Similarly to the VAMP2 chimera, SytI-ECFP was enriched at synaptic sites, identified by both exogenous SypI-ECFP and endogenous SV2. Upon treatment with α-Ltx, SytI-ECFP present in puncta

was translocated to the plasma membrane as a result of exocytosis, thus indicating that a certain amount of the exogenous protein is delivered to functional SVs (not shown).

We next studied whether SypI could control the sorting of VAMP1, a VAMP family member highly homologous to VAMP2 and expressed on SVs. The cytosolic N-terminal portion of VAMP1 was fused to ECFP (Figure 3.1), and the resulting chimera (ECFP-VAMP1) was overexpressed in neurons together with soluble EYFP. ECFP-VAMP1 showed a diffuse pattern of localization along the axons, with enrichments at the level of synaptic sites stained by SV2 (not shown). A virtually identical pattern of distribution was detected when ECFP-VAMP1 was transfected in neurons together with SypI-EYFP in a 1:1 ratio (Figure 3.7B and B').

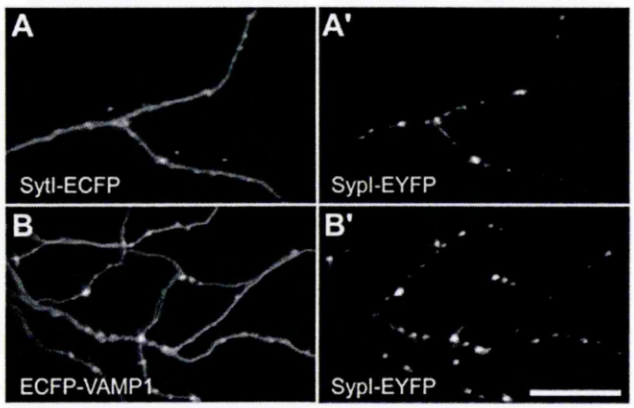

Figure 3.7. The sorting of SytI and VAMP1 is unaffected by overexpression of SypI Hippocampal neurons (15 DIV) co-transfected with the expression vectors for SypI-EYFP (A'-B') and either SytI-ECFP (A) or ECFP-VAMP1 (B) in a 1:1 ratio. Both SytI-ECFP (A) and ECFP-VAMP1 (B) show a diffuse extra-synaptic distribution along the axon of transfected cells, although they appear enriched at the level of synaptic puncta, where SypI-EYFP is concentrated.
Bar, 10 μm.

3.1.6 The amino-terminal portion of VAMP2 is required for the interaction with Synaptophysin I

The specificity of the SV targeting effect of SypI for VAMP2 is likely to depend on the formation of VAMP2-SypI heterodimers previously reported to occur in SVs from brain homogenates and hypothesized to play a regulatory role in exocytosis (Washbourne *et*

al., 1995). The specificity of this interaction was tested in SVs purified from rat brain and cross-linked by treatment with DSS. In these samples, both SypI and VAMP2 homo-dimers as well as SypI-VAMP2 hetero-dimers could be visualized, whereas SypI-VAMP1 heterodimers were not detected (Figure 3.8A). The formation of the VAMP2-SypI complex was detected biochemically also in cultured hippocampal neurons after protein cross-linking followed by immunoprecipitation with either anti-VAMP2 or anti-SypI antibodies and immunoblotting (Figure 3.8B).

VAMP1 and VAMP2 sequences maximally diverge in their proline-rich amino-terminal portions, which in the case of VAMP2 has been shown to be required for its interaction with SypI (Washbourne *et al.*, 1995; Bacci *et al.*, 2001). We examined in live cells whether the cytosolic N-terminal region of VAMP2, but not that of VAMP1, is required for the control of VAMP2 sorting by SypI. To this purpose, we swapped the N-terminal domains of VAMP2 and VAMP1 and fused the resulting constructs to ECFP and EYFP, generating the ECFP-VAMP2/CtVAMP1 and EYFP-VAMP1/CtVAMP2 chimeras (Figure 3.1). As expected, when transfected in hippocampal neurons together with soluble EYFP or ECFP, the two chimeras showed a diffuse pattern of distribution (not shown). When ECFP-VAMP2/CtVAMP1 or EYFP-VAMP1/CtVAMP2 were coexpressed in neurons together with either SypI-EYFP or SypI-ECFP respectively, ECFP-VAMP2/CtVAMP1 displayed a synaptic distribution, whereas EYFP-VAMP1/CtVAMP2 showed a diffused distribution (Figure 3.8C), indicating that the N-terminal portion of VAMP2 is necessary for its recruitment to synaptic sites by SypI.

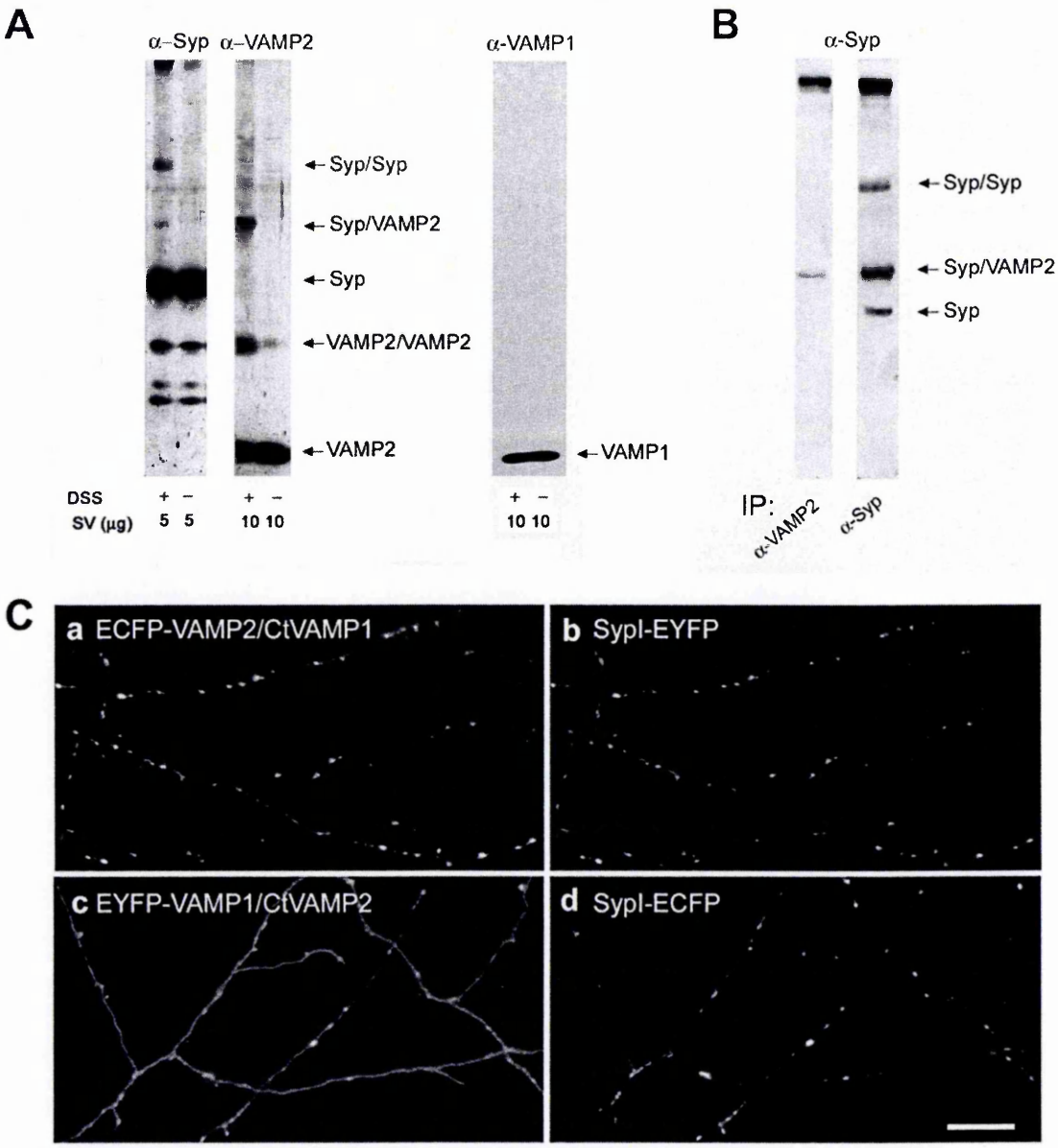

Figure 3.8. The interaction of SypI with the cytoplasmic tail of VAMP2 is required for the synaptic sorting of VAMP2.

(A) Purified SVs from rat brain were treated with the cross-linker DSS where indicated. Protein extracts were analyzed by Western Blotting and probed with anti-SypI and either anti-VAMP2 or anti-VAMP1 antibodies. Both SypI and VAMP2 homo-dimers and SypI-VAMP2 hetero-oligomers are visible. Virtually no SypI-VAMP1 hetero-dimers are detected. (B) SypI and VAMP2 interact with each other in mature hippocampal neurons. Cells were treated with the cross-linker DSS and processed for immunoprecipitation with either anti-SypI or anti-VAMP2 antibodies. Western Blotting with anti-SypI antibodies reveals the presence of SypI-VAMP2 heterodimers as well as SypI monomers and dimers. (C) ECFP-VAMP2/CtVAMP1 (a) and EYFP-VAMP1/CtVAMP2 (c) were transfected together with either SypI-EYFP (b) or SypI-ECFP (d) in a 1:1 ratio. While ECFP-VAMP2/CtVAMP1 shows a synaptic distribution, EYFP-VAMP1/CtVAMP2 displays a diffuse extra-synaptic distribution.
Bar, 10 µm.

3.1.7 Dynamics of Synaptophysin I/VAMP2 interaction during exocytosis

In order to study the dynamics of SypI and VAMP2 interaction at high spatial resolution during the exocytotic process, we measured the fluorescence resonance energy transfer (FRET) in living hippocampal neurons.

Exocytosis of SVs was stimulated by Taipoxin (Tpx), a snake neurotoxin endowed with phospholipase A_2 activity (Kamenskaya and Thesleff, 1974; Fohlman et al., 1976; Kini, 2003; Schiavo et al., 2000).

Neurons at 15 DIV were treated with 6 nM purified Tpx for 30 min in Ca^{2+}-containing medium (KRH). Videoanalysis showed that, after a few minute delay, the morphology of the axons changed progressively, and with time it assumed a characteristic bead-shaped structure, with the formation of discrete bulges (Figure 3.9A, left). Exposure to increasing concentrations of Tpx produced a dose-dependent increase in the percentage of swollen terminals [25 ± 6% (Tpx 6 nM), 60 ± 3% (Tpx 12 nM), 88 ± 3% (Tpx 24 nM) of the total boutons, n=950 per each condition]. Virtually no changes in the morphology of cell bodies were ever observed.

Similar bulges were recently reported to be induced by various snake presynaptic neurotoxins with phospholipase A_2 activity in several types of neurons (Rigoni et al., 2004) and they resemble the nerve terminal swelling induced by α-Ltx as a consequence of stimulation of massive exocytosis paralleled by impairment of endocytosis (Ceccarelli and Hurlbut, 1980; Valtorta et al., 1988; Pennuto et al., 2002). However, whereas α-Ltx was able to induce nerve terminal swelling when applied in the absence of extracellular Ca^{2+}, Tpx required the presence of extracellular Ca^{2+} in order to produce this effect (Figure 3.9A, left).

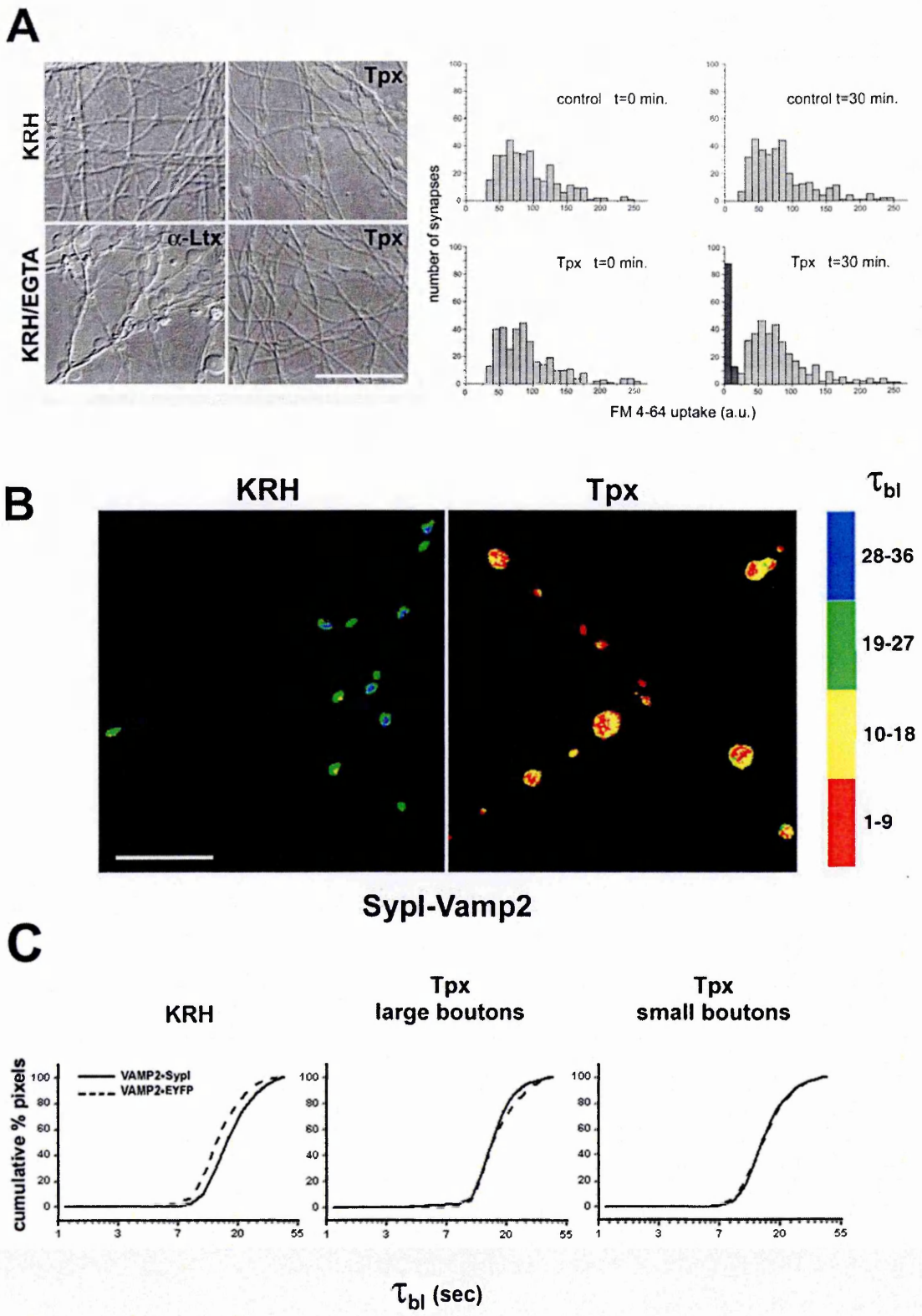

Figure 3.9 Spatial an d temporal dynamics of SypI-VAMP2 interaction during exocytosis.

(A) (Left) Ca^{2+}-dependence of the effects of Tpx and α-Ltx. Differential interference contrast (DIC) images of hippocampal neurons incubated for 30 min in either Ca^{2+}-containing (KRH) or Ca^{2+}-free (KRH/EGTA) medium in the presence or absence of either 6 nM Tpx or 0.1 nM α-Ltx. Nerve terminal swelling is induced by Tpx

exclusively in the presence of Ca^{2+}, and by α-Ltx in the absence of Ca^{2+}. Bar, 10 μm. (Right) Tpx induces exhaustive SV exocytosis in the population of swollen synaptic boutons. Distribution of classes of FM4-64 fluorescence. Neurons were loaded with FM4-64 during a 60 s incubation in depolarizing solution. The intensity of dye uptake was measured in single synaptic boutons at time 0 (left panels) and after a 30 min incubation in KRH in either the presence (right panel, bottom) or absence (right panel, top) of 6 nM Tpx. FM4-64 fluorescence was measured separately for the two populations of swollen (dark grey) and normally sized (light grey) synaptic boutons. The amount of dye retained in the normally sized boutons of the Tpx-treated sample was comparable to the amount found in the resting terminals. On the contrary, after Tpx treatment the dye content of swollen synaptic boutons was comprised in the lowest classes of intensity (which were not represented in the control samples), indicating that approximately all of the dye had been released from these terminals.
(B) Pseudocolor representation of the distribution of the time constants of donor photobleaching (τ_{bl}) measured in hippocampal neurons co-transfected with the expression vectors encoding for ECFP-VAMP2 and SypI-EYFP. Under resting conditions, the τ_{bl} values ranged from 36 to 19 s, whereas after Tpx treatment they decreased (ranging from 1 to18) in both normally sized and swollen synaptic boutons. Scale bar, 5 μm.
(C) Cumulative distribution of the time constants of donor photobleaching from the same experiment showed in B. In the Tpx-treated samples, normally sized and swollen synaptic boutons were analyzed separately. The similarities of the curves obtained for the two types of boutons confirms that SypI and VAMP2 interact under resting condition and dissociate after Tpx-stimulation in both small and swollen (large) boutons.

In order to estimate the extent of SV exocytosis induced by Tpx, the fluorescent styryl dye FM4-64 (Betz et al., 1996) was loaded into SVs using high K^+ depolarization, in a well established protocol that labels the entire pool of recycling vesicles (Pyle et al., 2000). The amount of FM4-64 loaded in single synaptic boutons was compared to the amount remaining in the bouton after a 30 min incubation of the neurons in Ca^{2+}-containing medium in either the absence or presence of Tpx. After 30 min the dye content measured in the untreated terminals was comparable to the amount retained in the class of normally sized boutons of the Tpx-treated sample (mean intensity ± SD, 82.9 ± 44.4 in control synapses, 82.4 ± 45.5 in small Tpx-treated terminals, n=342), indicating that only a small fraction of the dye was released during the 30-min incubation compared to the amount initially loaded (mean intensity ± SD, 91.1 ± 43.4, n=684). In contrast, the swollen synaptic boutons had released virtually the whole of the loaded dye (mean intensity ± SD, 5.3 ± 3.1, n=101), implying that exhaustive SV fusion

had occurred in response to Tpx treatment in this class of nerve terminals. Analysis of the distribution of fluorescence intensity showed that in the taipoxin-treated samples a population of synaptic boutons with minimal fluorescence intensity was present. This population, which was absent in the control samples, could be entirely accounted for by swollen terminals (Figure 3.9A, right).

The *in vivo* study of the molecular interactions between the SV proteins SypI and VAMP2 was carried out by measuring FRET in transfected neurons. Neurons (3 DIV) were co-transfected with the fluorescent fusion proteins ECFP-VAMP2 (donor protein) and SypI-EYFP (acceptor protein) and FRET was measured at 15-18 DIV as donor photobleaching using time-lapse video-digital imaging, as described in detail recently (Pennuto et al., 2002). To discriminate FRET on a synapse-by-synapse basis, the average time constants of donor photobleaching (τ_{bl}) were visualized using a pseudocolor scale (Figure 3.9B). Under resting conditions, the time constants were essentially comprised between 19 s and 36 s for all single pixel values within all synaptic boutons. After exposure to Tpx, the large majority of pixels in both small and large boutons displayed time constants in the 1-18 s range.

Small and large boutons of Tpx-treated samples were analyzed separately. Remarkably, for both types of boutons the curves of the distribution of τ_{bl} were similar for samples transfected with ECFP-VAMP2 and SypI-EYFP or with ECFP-VAMP2 and soluble EYFP (Figure 3.9C). In synaptic boutons of untreated samples the FRET efficiency was calculated to be 17.64 ± 0.5%, indicating that in living neurons the two proteins were close to each other on the SV membrane. At variance, in Tpx-treated samples, FRET efficiencies were 0.2 ± 0.7% for small synaptic boutons and –6.49 ± 0.4% for large boutons. The negligible FRET efficiency observed in both swollen and small boutons implies that VAMP2 dissociates from SypI prior to Tpx-induced vesicle fusion, as previously reported for α-Ltx (Pennuto et al., 2002). The negative FRET efficiency

observed in large boutons indicates that, under these conditions, a somewhat better transfer occurs between ECFP-VAMP2 and soluble EYFP than between ECFP-VAMP2 and SypI-EYFP.

3.1.8 Synaptophysin I controls the sorting of VAMP2 in non-neuronal cells

In order to investigate whether the ability of SypI to direct the sorting of VAMP2 depends on specific properties of neurons, we tested whether this process could be reconstituted in non-neuronal cells. A fluorescent chimera in which EYFP was fused to the intralumenal domain of VAMP2 (VAMP2-EYFP) was ectopically expressed in transformed human epithelial cells (Hela cells). Transfected cells were surface-stained with an antibody which recognizes EYFP to detect plasma-membrane associated VAMP2-EYFP, that exposes intralumenal fluorescent protein when targeted to the cell surface (Figure 3.10A). The staining pattern determined by the antibody resembled that of VAMP2-EYFP, indicating that most of the exogenous VAMP2 was localized at the plasma membrane.

The immunoreactivity of surface-associated VAMP2-EYFP was measured and compared with the total fluorescence of the chimera in individual transfected cells. This analysis revealed a dose-dependent linear increase in the fraction of exogenous VAMP2 targeted to the plasma membrane (Figure 3.10B). Confocal imaging of VAMP2-EYFP expressing cells showed that the chimera was present on both TfR-positive recycling endosomes, and early endosomes labelled by exogenous ECFP-Rab5, likely reflecting its constitutive recycling route (Figure 3.10C).

To obtain a direct visualization of the localization of VAMP2-EYFP at the plasma membrane, Hela cells were co-transfected to express VAMP2-EYFP together with a variant of ECFP bearing a farnesylation signal (CFP-F) which directs the protein to the plasma membrane. The pattern of fluorescence of VAMP2-EYFP and farnesylated ECFP were largely superimposed, consistent with the surface targeting of exogenous VAMP2 (Figure 3.10D).

An analogous observation was made when the chimera between synaptotagmin I and EYFP (SytI-EYFP) was co-expressed in Hela cells together with CFP-F. Also in this case the fluorescence pattern of SytI-EYFP and CFP-F were highly overlapped, indicating that exogenous SytI was predominantly associated with the plasma membrane. At variance, the distribution of exogenous SypI fused to EYFP (SypI-EYFP) was very dissimilar with respect to the distribution of CFP-F. SypI-EYFP was targeted to intracellular compartments and no co-localization with plasma membrane-associated CFP-F was visible (Figure 3.10D).

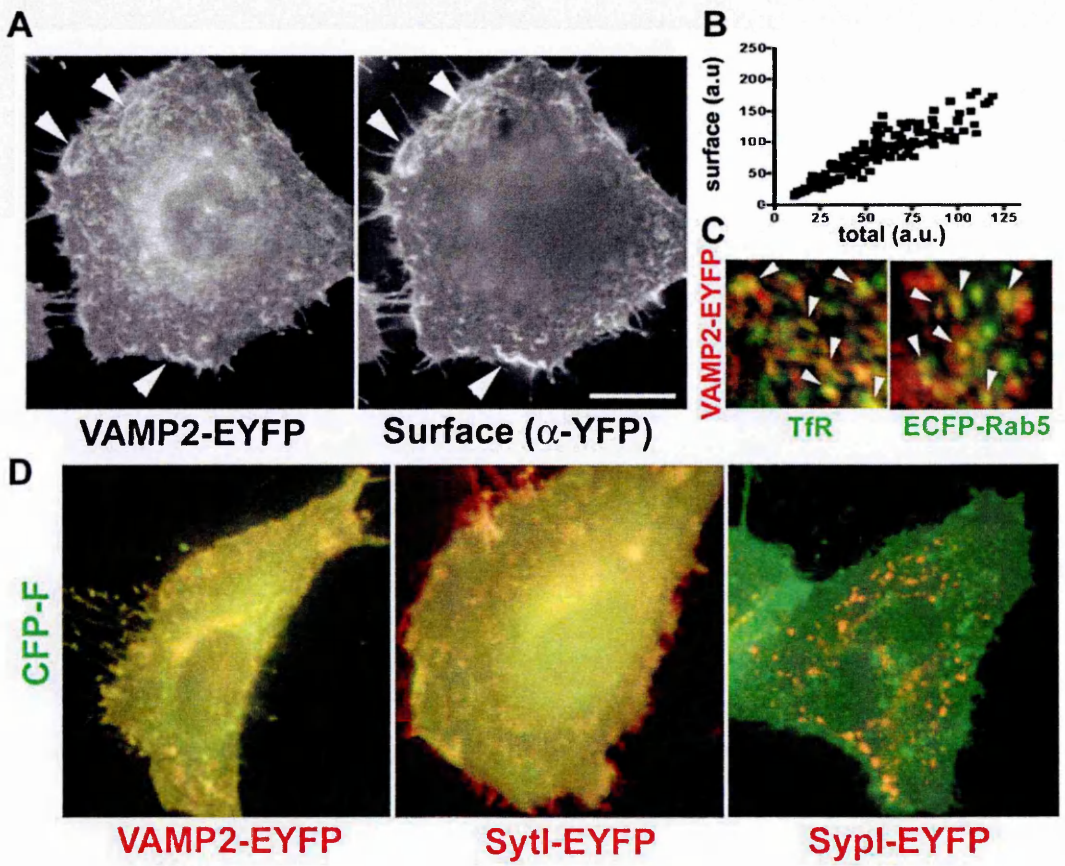

Figure 3.10. Differential targeting of exogenous SV proteins in Hela cells.
(A) Hela cells expressing the VAMP2-EYFP chimera, in which EYFP is fused to the intravesicular domain of the protein (Left) surface staining with an antibody which recognizes the fluorescent tag (Right). Note the co-localization of the two signals at the level of plasma membrane-associated ruffles (arrowheads). In the surface staining image the cell centre was out of the focus selected for imaging of the total VAMP2-EYFP fluorescence. (B) Surface staining of VAMP2-EYFP plotted against the total fluorescence of the chimera in individual cells. Each of the dots corresponds to a single cell. (C) VAMP2-EYFP-positive vesicles (red) show partial colocalization with either the TfR or ECFP-Rab5 (green) (arrowheads). (D) The plasma membrane marker

Farnesylated ECFP (CFP-F, green) co-expressed with either VAMP2-EYFP (Left), SytI-EYFP (Middle) or SypI-EYFP (Right) (red). Ectopically expressed SypI is localized to intracellular compartments while VAMP2 and SytI are mainly targeted to the plasma membrane. Bar, 10 μm in A,D and 2.5 μm in C.

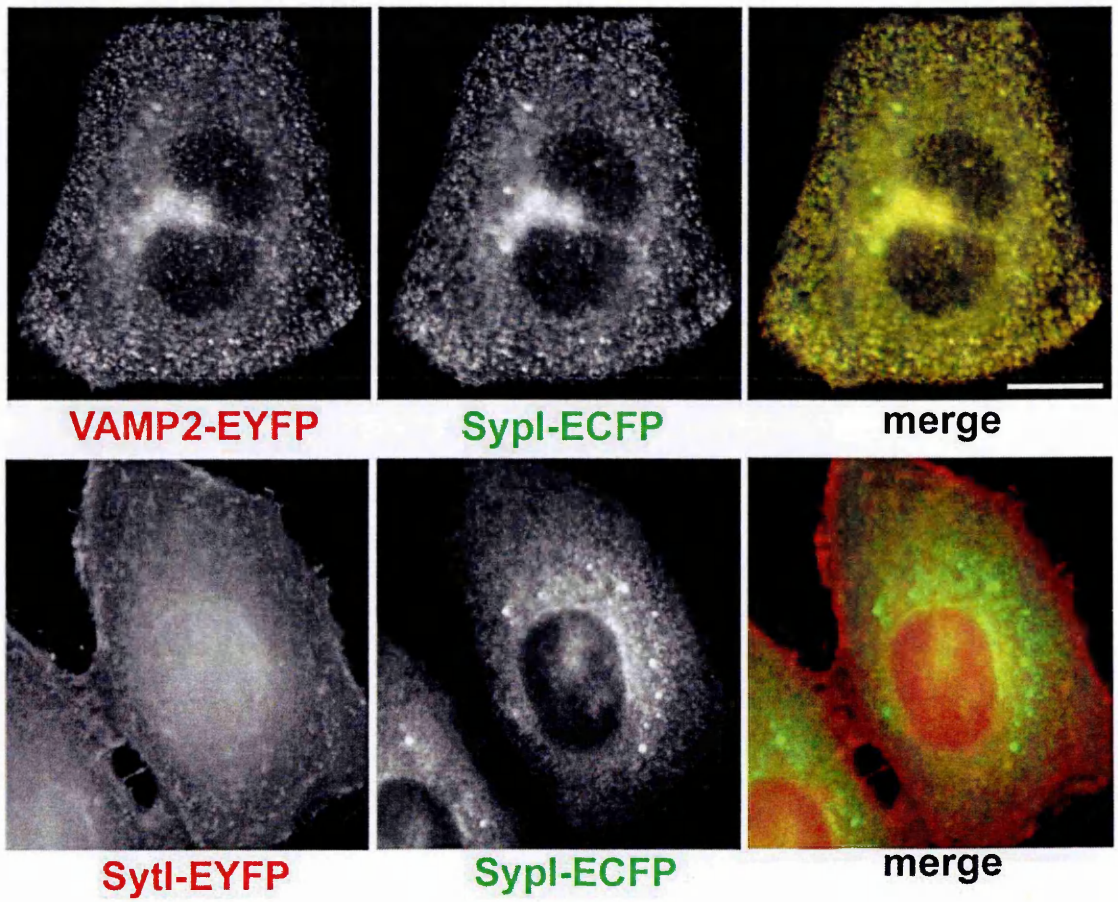

Figure 3.11. Synaptophysin I selectively controls the sorting of VAMP2 in Hela cells.
Hela cells co-expressing SypI-ECFP (green in the merged images) and either VAMP2-EYFP (Upper panel) or SytI-EYFP (Lower panel) (red in the merged images). Upon co-expression with SypI-ECFP, VAMP2-EYFP is directed to intracellular compartments whereas SytI-EYFP retains a plasma membrane localization. Bar, 10 μm

Remarkably, a dramatic change in the distribution of exogenous VAMP2 occurred upon co-expression with SypI in Hela cells. When expressed together with SypI-ECFP, VAMP2-EYFP no longer accumulated at the plasma membrane but was selectively targeted to intracellular compartments where it colocalized with exogenous SypI. At variance, exogenous synaptotagmin I (SytI-EYFP) retained a prominent plasma membrane targeting when co-expressed with SypI-ECFP (Figure 3.11). Thus, SypI exerts a selective effect on VAMP2 sorting in non-neuronal cells causing the

redistribution of exogenous VAMP2 from the plasma membrane to intracellular compartments. This effect does not appear to be specific for Hela cells since it was observed in transformed fibroblasts and epithelial cells of different origins, as well as in primary cultures of astrocytes derived from the rat cortex (data not shown).

In non-neuronal cells, ectopically expressed SypI accumulates in endosomes positive for the transferrin receptor (i.e., recycling endosomes) (Figure 3.12A; Cameron et al, 1991 and references within). Interestingly, when expressed at high doses exogenous SypI induced a profound alteration in the morphology of these organelles, which appear greatly enlarged (compare transferrin receptor-positive puncta in either SypI-EYFP-transfected or untransfected cells in Figure 3.12A). The appearance of enlarged recycling endosomes is accompanied by a reduction in the number of SypI-positive organelles. Enlarged SypI-containing recycling ensodomes retained their molecular identity, since they were positive for neither early-endosome antigen 1 nor lysosome-associated membrane protein 1 (Figure 3.12A). The Golgi apparatus was not affected by SypI-EYFP expression, as shown by staining for the specific marker Giantin, thus ruling out the possibility that enlarged endosomes had originated by the fragmentation of the Golgi cisternae (Figure 3.12A). Importantly, labelling of SypI-EYFP expressing cells with filipin revealed a preferential accumulation of cholesterol at the level of SypI-positive enlarged endosomes.

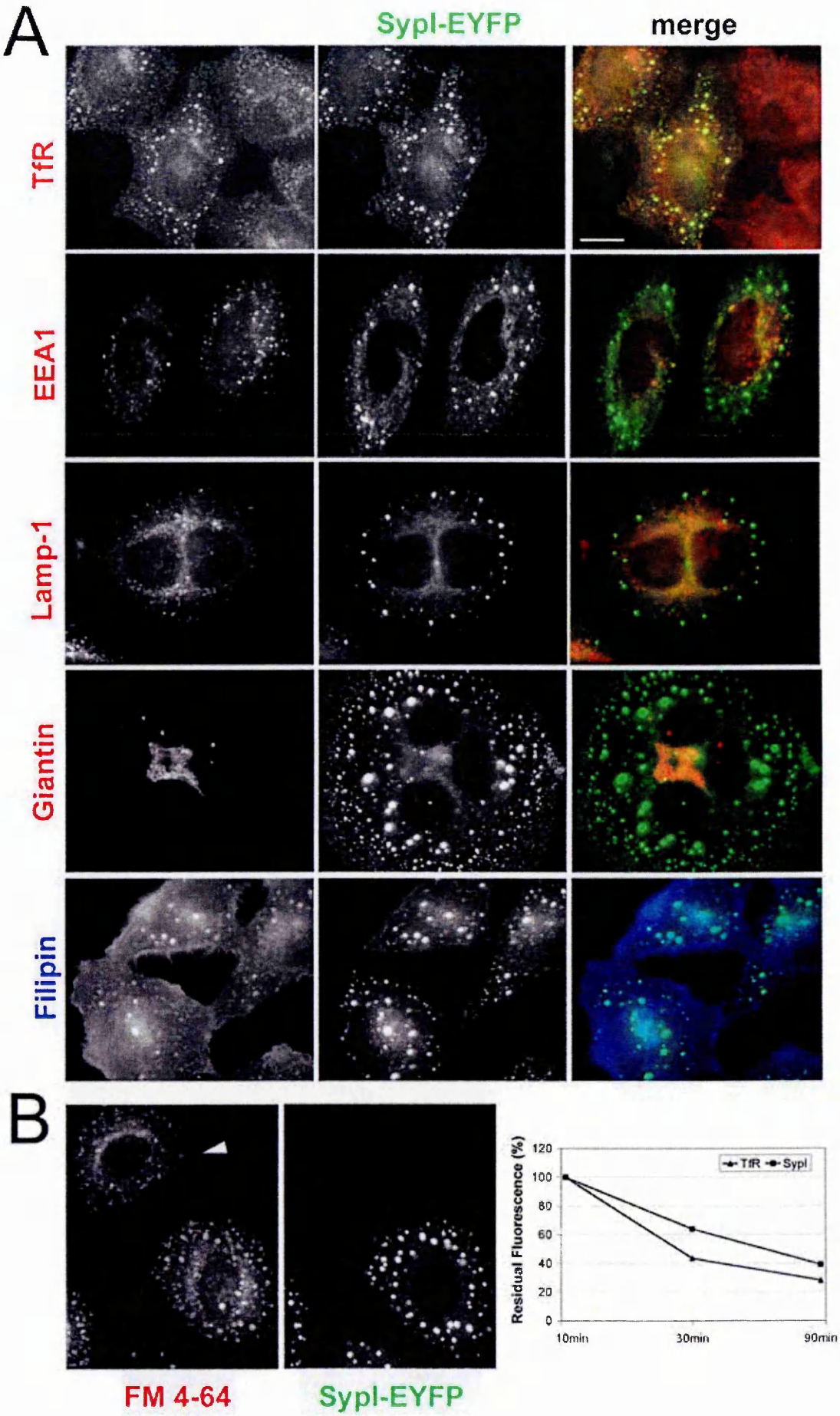
A
Sypl-EYFP
merge
TfR
EEA1
Lamp-1
Giantin
Filipin
B
FM 4-64
Sypl-EYFP
Residual Fluorescence (%)
120
100
80
60
40
20
0
TfR
Sypl
10min
30min
90min

Figure 3.12. High doses of exogenous Synaptophysin I alter both the morphology and the recycling properties of transferrin receptor-positive endosomes without affecting their molecular identity.
(A) Hela cells expressing SypI-EYFP at high doses (green in the merged images) were stained for endogenous transferrin receptor (TfR), early endosome antigen 1 (EEA1), lysosome-associated membrane protein 1 (Lamp-1), Giantin (Red in the merged images) or with filipin to visualize cholesterol (blue in the merged image). SypI-EYFP selectively affects the morphology of transferrin receptor-positive recycling endosomes (B) (Left) Cells expressing SypI-EYFP were incubated with the fluorescent dye FM 4-64 for 1 min in resting solution (KRH) followed by 2 min washing in KRH. The arrowhead points to an untransfected cell which had internalized the dye. (Right) Percent reduction in the FM 4-64 fluorescence associated with intracellular compartments positive for either SypI-EYFP (SypI, squares) or TfR-EYFP (TfR, triangles) calculated 30 and 90 min after FM 4-64 loading relative to the fluorescence measured 10 min after loading. Membrane recycling is slower in the enlarged endosomes positive for exogenous SypI. The mean values from two independent experiments are reported. Standard deviations are too small to be visible in this plot. Bar, 10 μm.

To study membrane recycling through SypI-positive endosomes we used the styryl dye FM 4-64, which is internalized upon endocytosis. After a brief (1 min) incubation of cells expressing SypI-EYFP with the dye, fluorescent staining was associated with enlarged SypI-containing endosomes of transfected cells, as well as endocytic compartments of non-transfected cells (Figure 3.12B, left). The kinetics of membrane recycling were analyzed by measuring FM 4-64 fluorescence 10, 30 and 90 min after loading of the dye in either SypI-positive enlarged endosomes of SypI-EYFP expressing cells or transferrin receptor-positive endosomes of TfR-EYFP expressing cells. The rate of dye unloading was reduced in SypI-containing enlarged endosomes as compared to recycling endosomes containing exogenous TfR (Figure 3.12B, right). Thus, when expressed at high doses exogenous SypI causes an impairment in both the structure and the recycling properties of TfR positive endosomes.

In order to gain a quantitative evaluation of the effect of SypI on VAMP2 sorting in non-neuronal cells, Hela cells were co-transfected with plasmids expressing SypI-ECFP and VAMP2-EYFP, and surface stained with an antibody which recognizes EYFP exposed by plasma membrane-associated VAMP2-EYFP. Figure 3.13A shows cells

expressing various levels of SypI-ECFP. Despite the expression of comparable levels of VAMP2-EYFP in these cells, the amount of chimera targeted to the plasma membrane, as revealed by surface-immunoreactivity, was apparently higher in cells expressing lower levels of SypI-ECFP. The ratio between surface-associated to total VAMP2-EYFP fluorescence was measured and correlated to SypI-ECFP fluorescence in individual cells. This analysis showed a proportionate reduction in the amount of plasma membrane-associated VAMP2-EYFP with increasing levels of exogenous SypI.

To test whether the long carboxy-terminal domain of SypI was implicated in its ability to control the sorting of exogenous VAMP2, cells co-expressing VAMP2-EYFP with a carboxy-terminal truncated version of SypI fused to ECFP (SypIΔC-ECFP) were stained to detect surface associated VAMP2-EYFP (Figure 3.13C). The distribution of the SypIΔC-ECFP mutant was reminiscent of the distribution of the full length SypI, with a preferential targeting to intracellular compartments which also contained exogenous VAMP2. Although expression of SypIΔC-ECFP caused a modest, but reproducible, decrease in the amount of surface-associated VAMP2-EYFP, this effect was significantly weaker than the effect produced by wild type SypI (Figure 3.13B). Thus, the carboxy-terminal domain of SypI is required to achieve an efficient regulation of VAMP2 sorting in Hela cells.

Overexpression of the recycling endosome-associated protein Rab11 causes endosome enlargement accompanied by accumulation of free cholesterol in these compartments (Hölttä-Vuori et al., 2002), an effect similar to that produced by high doses of SypI. Hela cells co-expressing VAMP2-EYFP and Rab11 fused to ECFP (ECFP-Rab11) were stained to detect surface-associated VAMP2-EYFP (Figure 3.13C). Exogenous VAMP2 was present in Rab11-positive recycling endosomes. However, the ratio between surface-associated and total VAMP2-EYFP was only modestly reduced by Rab11

expression and was significantly higher as compared to the ratio measured upon SypI expression (Figure 3.13B).

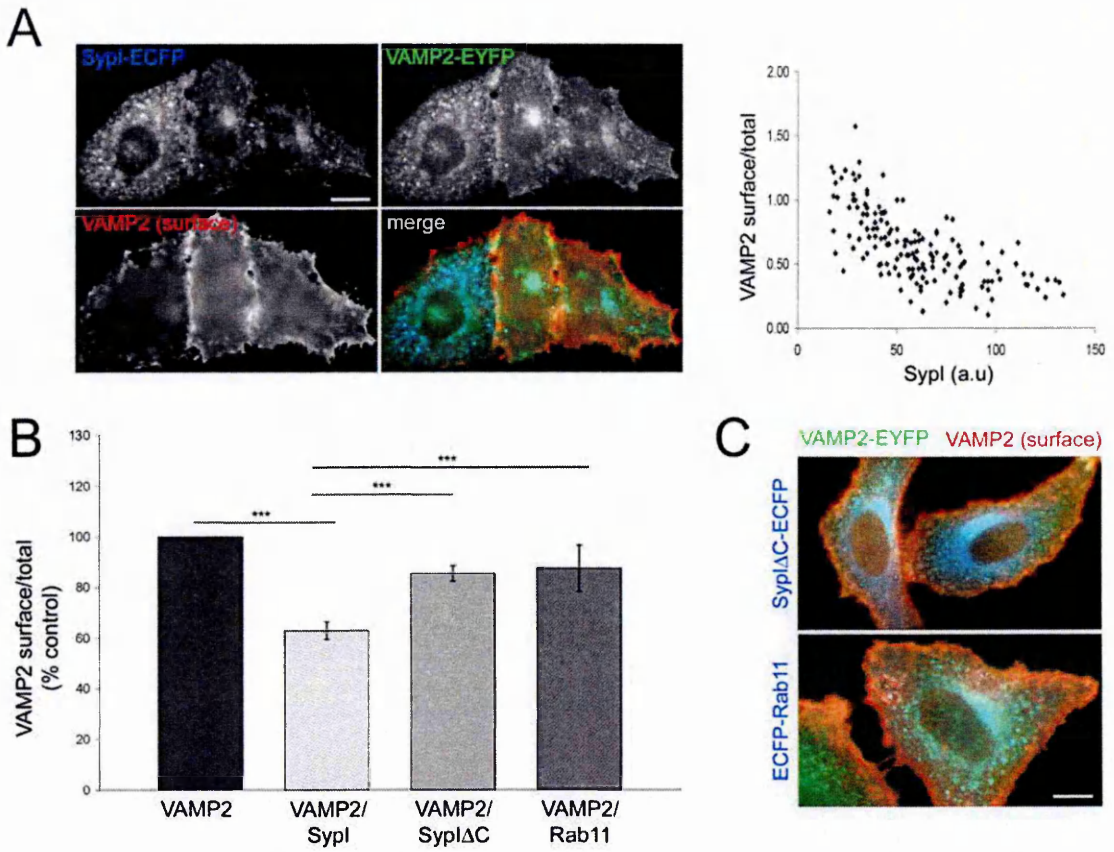

Figure 3.13. Synaptophysin I controls the sorting of VAMP2 in Hela cells.
(A) (Left) Hela cells co-expressing VAMP2-EYFP (green in the merged image) and SypI-ECFP (blue in the merged image) surface stained with an anti-GFP antibody (red in the merged image) to detect the fraction of VAMP2-EYFP associated with the plasma membrane. (Right) The ratio between surface and total VAMP2-EYFP fluorescence is plotted against the levels of SypI-ECFP fluorescence in individual cells. Each of the dots corresponds to a single cell. Cells were transfected with VAMP2-EYFP and SypI-ECFP-expressing plasmids in either 1:1 or 1:3 ratio in order to increase variability in the levels of the two chimeras. (B) Percent changes in the ratio between surface and total VAMP2-EYFP fluorescence in Hela cells co-expressing VAMP2-EYFP and either SypI-ECFP (VAMP2/SypI), carboxy-terminal truncated SypI-ECFP (VAMP2/SypIΔC), or ECFP-Rab11 (VAMP2/Rab11) with respect to cells co-expressing VAMP2-EYFP and soluble ECFP (VAMP2). In all the conditions plasmids were transfected at equimolar concentrations. The mean (± SD) from 2-5 independent experiments is reported. 50-150 cells were analyzed in each experiment. *** $p < 0.001$ (Student's *t* test). (C) Hela cells co-expressing VAMP2-EYFP (green) and either SypIΔC-ECFP or ECFP-Rab11 (blue) surface stained to detect plasma membrane-associated VAMP2-EYFP (red).
Synaptophysin I exerts a dose-dependent and selective effect on VAMP2 sorting in Hela cells. This effect is largely mediated by the carboxy-terminal domain of synaptophysin I. Bar, 10 μm

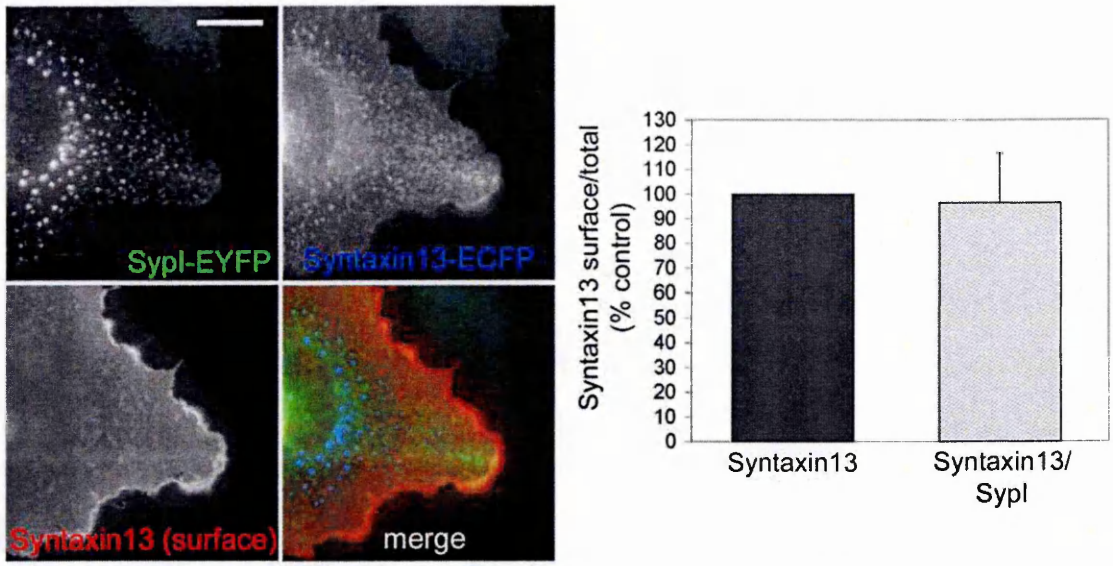

Figure 3.14. Synaptophysin I does not affect the plasma membrane targeting of exogenous Syntaxin 13 in Hela cells.
(Left) Hela cells co-expressing syntaxin13-ECFP (green in the merged image) and SypI-EYFP (blue in the merged image) at equimolar concentration, surface stained with an anti-GFP antibody (red in the merged image) to detect plasma membrane-associated syntaxin13-ECFP. Bar, 10 μm (Right) Percent changes in the ratio between surface and total syntaxin13-ECFP fluorescence in Hela cells co-expressing syntaxin13-ECFP and SypI-EYFP (Syntaxin13/SypI) with respect to cells co-expressing syntaxin13-ECFP and soluble EYFP (Syntaxin13). The mean (± SD) is reported (140 cells from 2 independent experiments). p=0.9 (Student's *t* test versus control).

In order to assess whether SypI cause a non-selective perturbation of the sorting of proteins trafficked through transferrin receptor-positive recycling endosomes, ECFP was fused to the intralumenal carboxy-terminal tail of the single-pass protein syntaxin 13, that in non-polarized cells is found primarily in early and recycling endosomes, where it colocalizes with the transferrin receptor (Prekeris et al., 1998). Cells co-expressing the syntaxin13-ECFP chimera together with SypI-EYFP were surface stained with an antibody which recognizes ECFP exposed to the extracellular surface following syntaxin13-ECFP targeting to the plasma membrane. The ratio between surface-associated immunoreactivity and total syntaxin13-EYFP fluorescence did not vary upon SypI-ECFP expression (Figure 3.14). Thus, the effect of SypI on protein

sorting is specific for VAMP2 and is not due to a general impairment of protein trafficking through recycling compartments.

The specificity of the effect of SypI on VAMP2 sorting may rely on the formation of VAMP2-SypI hetero-dimers. The formation of the VAMP2-SypI complex was detected biochemically in Hela cells co-expressing exogenous VAMP2 and HA-tagged SypI by immunoprecipitation with anti HA-antibody and immunoblotting with anti-SypI and anti-VAMP2 antibodies (Figure 3.15). Co-immunoprecipitation also revealed the formation of hetero-complexes between VAMP2 and the carboxy-terminal truncated SypIΔC ectopically expressed in Hela cells. Thus, the carboxy-terminal domain of SypI, which is required for efficient regulation of VAMP2 sorting, is dispensable for the SypI-VAMP2 interaction.

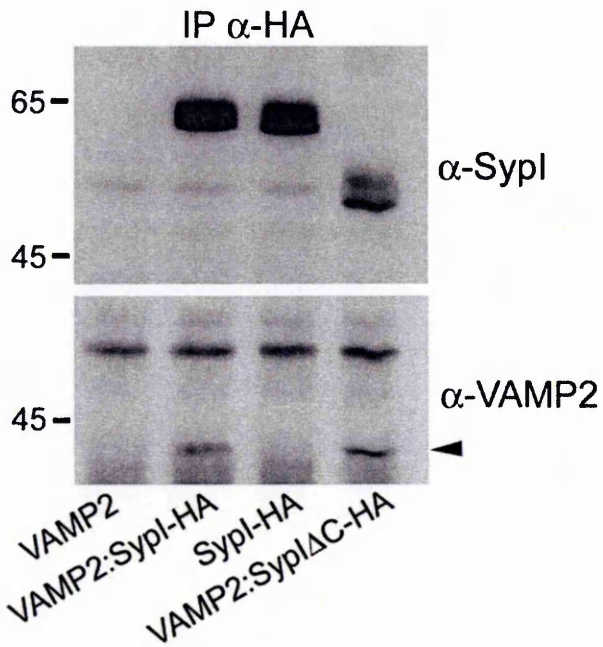

Figure 3.15. SypI and VAMP2 interact with each other in Hela cells. Hela cells co-expressing either VAMP2-EYFP and soluble ECFP (VAMP2), VAMP2-EYFP and an HA-tagged SypI-ECFP chimera (VAMP2:SypI-HA), SypI-HA and soluble ECFP (SypI-HA), or VAMP2-EYFP and HA-tagged carboxy-terminal truncated SypIΔC-ECFP chimera (VAMP2: SypIΔC-HA) were processed for immunoprecipitation with an anti-HA antibody. Western Blotting analysis with an anti-VAMP2 antibody reveals the presence of hetero-complexes between VAMP2-EYFP (arrowhead) and either SypI-HA or SypIΔC-HA, visualized by an anti-SypI antibody. Bands with molecular weight higher than 45 KDa present in all the lanes correspond to the light chains of the anti-HA antibody recognized by the anti-mouse secondary antibody exploited to detect the anti-VAMP2 antibody.

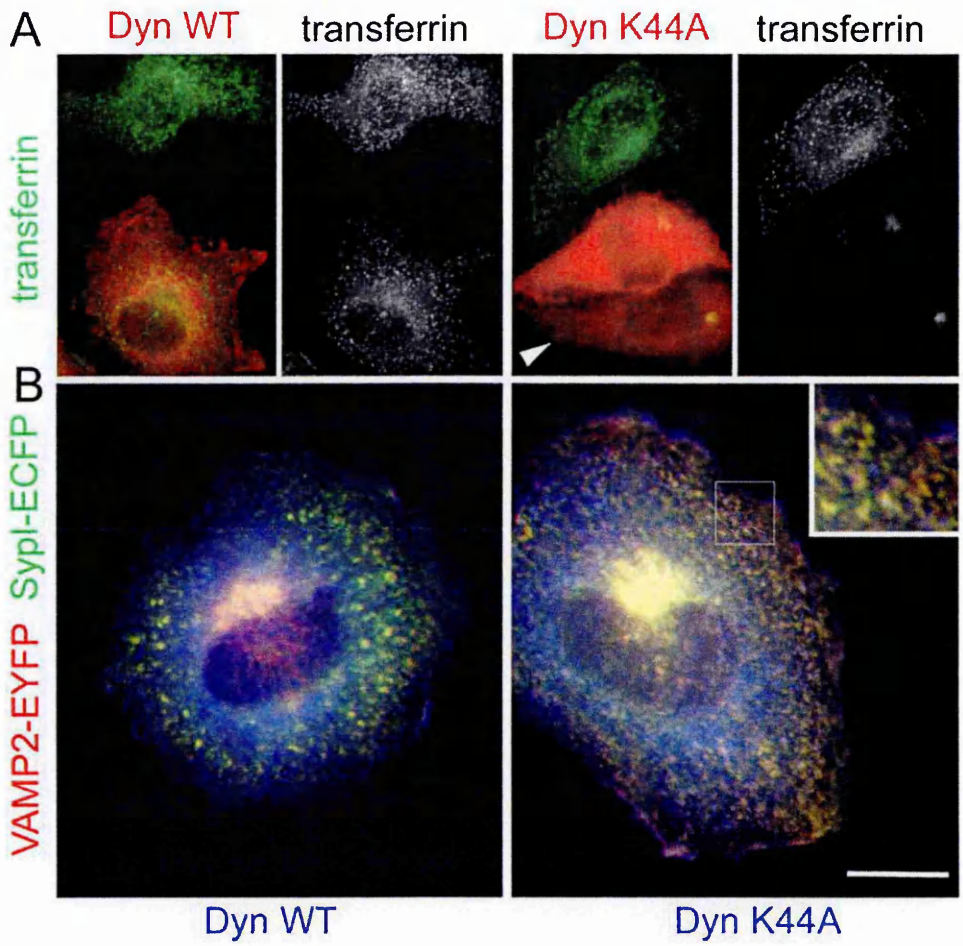

Figure 3.16. Endocytosis is dispensable for Synaptophysin I-directed sorting of exogenous VAMP2 in Hela cells.
(A) Hela cells expressing either HA-tagged wild type dynamin (Dyn WT) or dominant negative dynamin (Dyn K44A) were exposed to FITC-conjugated transferrin during a 30 min incubation, followed by acid stripping to remove surface-associated transferrin (green). Staining with an anti-HA antibody identifies cells expressing exogenous dynamins (red). Grayscale images show internalized transferrin extracted from the corresponding merged images. Dyn K44A effectively inhibits endocytosis even when expressed at low doses (arrowhead). (B) Hela cells triple-transfected to express VAMP2-EYFP (red), SypI-ECFP (green) and either wild type dynamin or the K44A mutant (blue). The inset shows an enlargement of the square-selected area. Note the co-localization of VAMP2-EYFP and SypI-ECFP in intracellular compartments in both cells expressing the wild type and mutant dynamin. Bar, 20 μm in A; 10 μm in B and 4 μm in the inset in B.

In order to test whether endocytosis from the plasma membrane is critical in order for SypI to control VAMP2 sorting, the dominant negative dynamin mutant bearing the

aminoacid substitution K44A (Dyn K44A) was exploited. Expression of Dyn K44A effectively abolished the internalization of fluorescein-conjugated transferrin compared to cells expressing wild type dynamin. (Figure 3.16A). I sought to investigate whether the ability of SypI to recruit VAMP2 to intracellular compartments could be impaired by the block of endocytosis. Cells were triple transfected with the expression plasmids for VAMP2-EYFP, SypI-ECFP and either wild type dynamin or the K44A mutant in a ratio of 1:1:2 and analyzed 3 days after transfection. As expected, inhibition of endocytosis increased the plasma membrane localization of both exogenous SypI and VAMP2. However, VAMP2-EYFP was mainly associated with SypI-ECFP-positive intracellular compartments in both cells expressing wild type and K44A mutant dynamins (Figure 3.16B). This result shows that endocytosis is dispensable for SypI-directed sorting of exogenous VAMP2 in non-neuronal cells.

3.2 STUDY OF SYNAPTIC VESICLE DYNAMICS IN DEVELOPING NEURONS

3.2.1 SVs are clustered in the core of the growth cone by F-actin and recycle at slow rates

In order to monitor the dynamics of SV protein-containing organelles in living neurons, we exploited a lentiviral-mediated expression system (Lotti et al., 2002) to direct the expression of fluorescent chimeras of synaptophysin I (SypI) and VAMP2 fused to EYFP and ECFP, respectively. To limit the possibility of altered sorting of the exogenous proteins to organelles other than SVs, which is observed at high expression levels, the transgenes were placed under the control of a constitutive cellular promoter, the phosphoglycerol kinase (PGK) promoter.

SypI-EYFP was associated with vesicles trafficked along the axon and concentrated in the central (C) domain of the axonal growth cone, characterized by high organelle and microtubule density (Forscher et al., 1987). These vesicles appeared to be virtually absent from the peripheral (P) domain of the growth cone, characterized by low organelle density and high F-actin content (Figure 3.17a-b). Similarly, vesicles bearing endogenous SV2, a distinctive marker of SVs at mature synapses, were clustered in the core of the C domain but excluded from the P domain underlain by F-actin. In agreement with previous studies, we will refer to organelles containing SV proteins prior to the formation of synaptic contacts as *bona fide* SVs (Matteoli et al., 1992; Sabo and McAllister, 2003).

To study membrane recycling in the growth cone we used the styryl dye FM 4-64 which is internalized upon endocytosis. After a brief (1 min) incubation with the dye, intense fluorescent staining was associated with large membrane structures at the distal edge of the C domain. However, virtually no SypI-positive vesicles contained FM 4-64,

indicating a negligible rate of basal SV recycling, when compared to the high levels of constitutive endocytosis observed in the growth cone at this developmental stage (Figure 3.17c and c').

In order to answer the question as to whether F-actin plays a role in the retention of SVs in the C domain, we exposed hippocampal neurons to cytochalasin D, which causes F-actin disassembly (Forscher and Smith, 1988; Contestabile et al., 2003). Upon such treatment, SVs marked by either SypI-EYFP or endogenous SypI were dispersed throughout the growth cone and reached the distal edge of the P domain (Figure 3.17 d and data not shown). Thus, disorganization of actin allows the movement of SVs into the P domain.

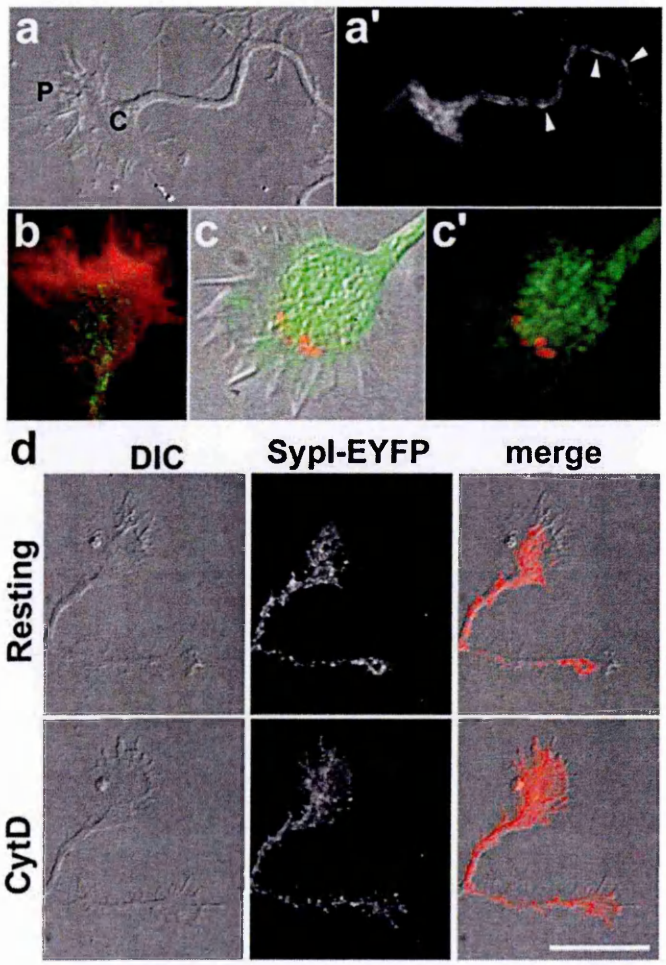

Figure 3.17. Localization of SVs in the core domain of the growth cone depends on the integrity of the F-actin meshwork.
DIC (a) and fluorescence (a') images of a living rat hippocampal neuron expressing SypI-EYFP. The chimera is associated with organelles distributed along the axon (arrowheads in a') and concentrated in the central domain of the growth cone (C), but virtually absent in the peripheral domain (P). (b) Immunofluorescence of a growth cone

labeled with an antibody against the SV protein SV2 and TRITC-conjugated phalloidin to stain F-actin. Vesicles bearing endogenous SV2 (green) are clustered in the core of the growth cone and excluded from the P domain enriched in F-actin (red). (c and c') Basal uptake of FM 4-64 (red) in a growth cone incubated with the dye for 1 min, fixed and retrospectively labeled with an anti-SypI antibody (green). The overlay between the fluorescence (c') and DIC images is shown in c. FM 4-64 is internalized in large organelles at the interface between the C and P domains. (d) Growth cones of rat hippocampal neurons expressing SypI-EYFP were imaged before and after incubation with 10 μM cytochalasin D (CytD) for 15 min at RT. In the right column, the overlay between the DIC and fluorescence (red) images is shown. At rest, SypI-positive vesicles are concentrated in the C domain, but they disperse throughout the growth cone after cytochalasin D treatment. Bar, 10 μm in a and a', 8 μm in b, 6 μm in c and c', 16 μm in d.

3.2.2 Evidence for a developmental regulation of evoked SV recycling

When neurons at 2 DIV were incubated for 3 min under basal conditions in the presence of FM 4-64, the dye was internalized in a large compartment at the edge of the C domain, and could not be released upon a 1 min incubation in a high K^+-containing solution. Retrospective staining for endogenous VAMP2 showed that SVs had not become labeled by FM 4-64 during depolarization, thus indicating that no intermixing occurs between the pool of SVs and actively recycling compartments in the growth cone (Figure 3.18A, upper panel).

Neurons at 2 DIV expressing exogenous ECFP-VAMP2 were incubated in high K^+ for 1 min in the presence of the FM 4-64 to directly test the effect of depolarization on SV recycling in the growth cone at this developmental stage. The pattern of FM 4-64 fluorescence after depolarization was remarkably similar to the pattern observed after constitutive uptake of the dye during 1 min incubation in KRH. An intense FM 4-64 staining was associated with large membrane structures at the distal limit of the C domain. However, no SVs containing exogenous VAMP2 were labeled by the dye, indicating that SVs do not undergo exo-endocytosis upon depolarization at early developmental stages (Figure 3.18A, lower panel).

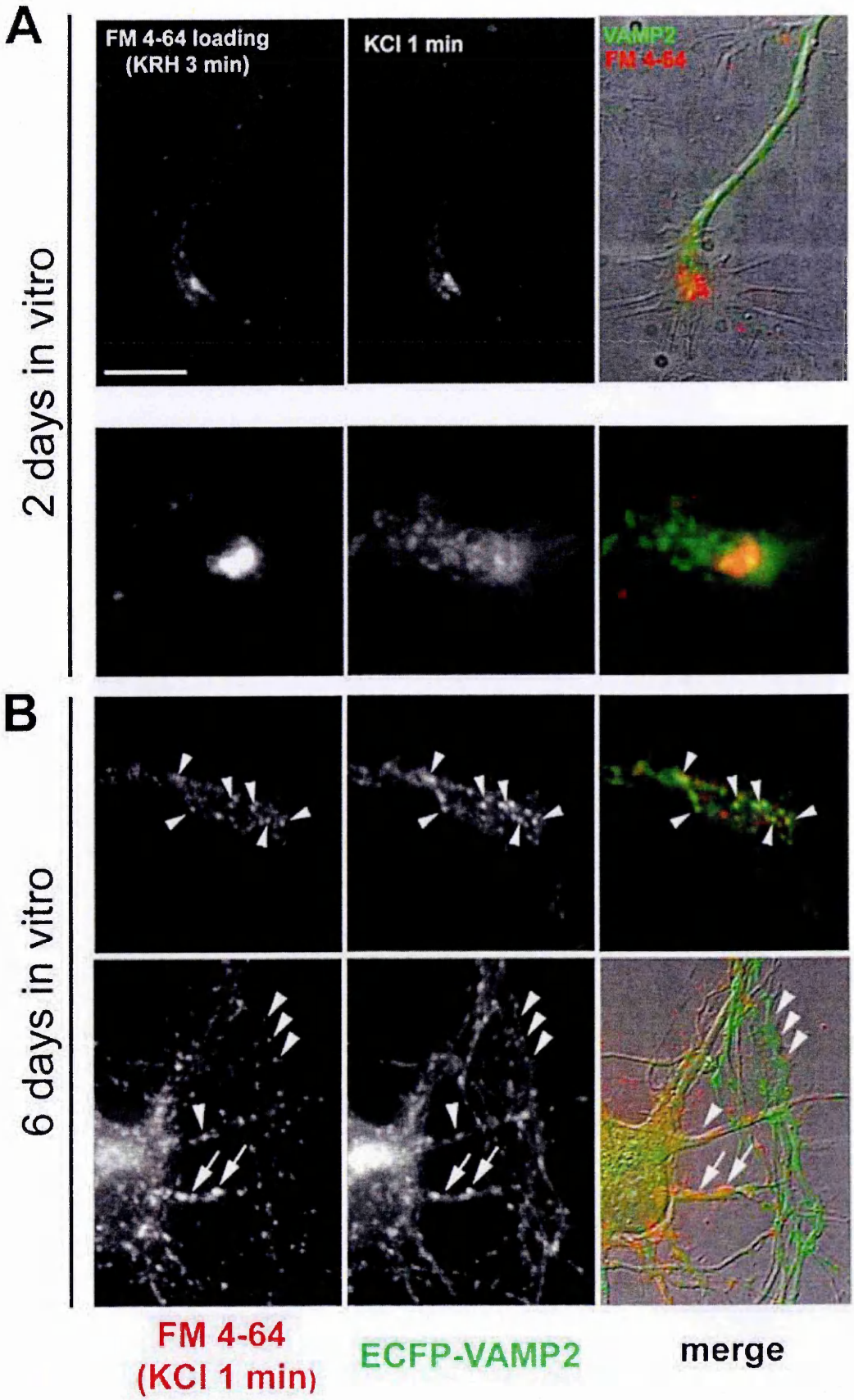
A
FM 4-64 loading
(KRH 3 min)
KCl 1 min
VAMP2
FM 4-64
2 days in vitro
B
6 days in vitro
FM 4-64
(KCl 1 min)
ECFP-VAMP2
merge

Figure 3.18. A developmental switch regulates depolarization-induced SV recycling in the growth cone.
(A) Rat hippocampal neurons at 2 days in vitro. (Upper panel) Uptake of FM 4-64 (red in the merged image) in a growth cone incubated with the dye for 3 min in resting solution (Left), followed by incubation with a high K^+-depolarizing solution for 1 min (Middle). After depolarization the growth cone was fixed and retrospectively labelled with an anti-VAMP2 antibody (green) (Right). The overlay between the fluorescence and DIC images is shown. (Lower panel) Growth cone of a neuron expressing ECFP-VAMP2 (green in the merge image) incubated with an high K^+-depolarizing solution for 1 min in the presence of FM 4-64 (red in the merged image). VAMP2-positive vesicles do not internalize FM 4-64 upon depolarization.
(B) Rat hippocampal neurons at 6 days in vitro. Neurons expressing ECFP-VAMP2 (green in the merged images) incubated with a high K^+-depolarizing solution for 1 min in the presence of FM 4-64 (red in the merged images). (Right) The overlay between the fluorescence and DIC images is shown. The upper panel shows an isolated growth cone whereas a growth cone which had contacted several neurites is shown in the lower panel. Arrowheads point to VAMP2-positive vesicles that internalized the dye upon depolarization. Note the presence of VAMP2-positive synaptic varicosities that internalized the dye (arrows). Bar, 10 μm in A (upper panel) and B (lower panel); 5.5 μm in A (lower panel) and B (upper panel).

Synaptic contacts begin to form at 6 DIV in cultured hippocampal neurons. Importantly, when neurons at 6 DIV were exposed to FM 4-64 during 1 min depolarization with high K^+, the dye was loaded in ECFP-VAMP2-positive SVs at both synapses (arrows in Figure 3.18B, lower panel) and isolated growth cones (arrowheads in Figure 3.18B, upper panel). Thus, SVs which appear to be reluctant to undergo both spontaneous and evoked exo-endocytosis at early developmental stages, become competent for depolarization-induced recycling at later stages.

3.2.3 cAMP-modulated control of the distribution and recycling of SVs in the growth cone

As cAMP was previously shown to affect organelle trafficking in the growth cone (Forscher et al., 1987; Hollenbeck, 1993), we wanted to determine whether cAMP-dependent pathways have a role in the control of the distribution of SVs in the C domain. Neurons expressing SypI-EYFP were imaged before and after incubation with forskolin, an adenylate cyclase activator which increases intracellular cAMP levels

(Seamon et al., 1981). This treatment markedly altered SV distribution, by inducing dispersion of SVs from the C domain throughout the growth cone. The changes in SV distribution occurred in the absence of major rearrangements in growth cone morphology, as shown by differential interference contrast (DIC) images (Figure 3.19, upper panel). Treatment of neurons with the membrane-permeant and phosphodiesterase-resistant cAMP analog BT-cAMP (10 mM) for 7 min produced a comparable dispersion of SVs in growth cones (data not shown). In most of the growth cones analyzed, we also observed a reduction in the size of the fluorescent puncta representing SV clusters, suggesting a decrease in SV aggregation.

The specificity of the effect of cAMP increase on the localization of SVs was tested by staining neurons treated with forskolin for the integral SV protein synaptotagmin I (Koh and Bellen, 2003), the endosomal antigen syntaxin 13 (Prekeris et al., 1999) and F-actin. Prior to forskolin treatment, both synaptotagmin I- and syntaxin 13-labeled vesicles were localized in the C domain and excluded from the P domain. Forskolin induced mobilization of synaptotagmin I-positive SVs into the P domain, whereas syntaxin 13-positive endosomes remained confined to the C domain (Figure 3.19, lower panel). F-actin staining was unaffected [mean phalloidin fluorescence ± SD: 90 ± 5.4 and 101 ± 2.6 (a.u.) in resting and forskolin-treated growth cones, respectively; n=30].

As SVs diffused towards the growth cone membrane upon forskolin treatment, we asked whether the rate of basal SV recycling was also modulated by intracellular cAMP levels. To accurately study exo-endocytosis of SVs occurring at low rates in the growth cone, we exploited an assay based on the uptake of an antibody directed against the lumenal domain of synaptotagmin I (Syt_L). This antibody binds to synaptotagmin I following exposure of the SV membrane at the cell surface and is then internalized by endocytosis of the SV membrane (Matteoli et al., 1992). Neurons at 2-3 DIV were incubated for 5 min with Syt_L in KRH in the presence or absence of forskolin, followed

by an additional 10 min incubation with the antibody in KRH. After treatment, neurons were counterstained with an antibody directed against the cytoplasmic domain of synaptotagmin I (Syt_C) and the levels of Syt_L uptake were normalized to the total synaptotagmin I content (i.e. Syt_C signal intensity) of each growth cone. Forskolin-induced SV mobilization was accompanied by a significant increase in the fraction of Syt_L internalized by the growth cones (Figure 3.20), indicating that cAMP-dependent pathways control the basal recycling of SVs in growth cones of isolated axons.

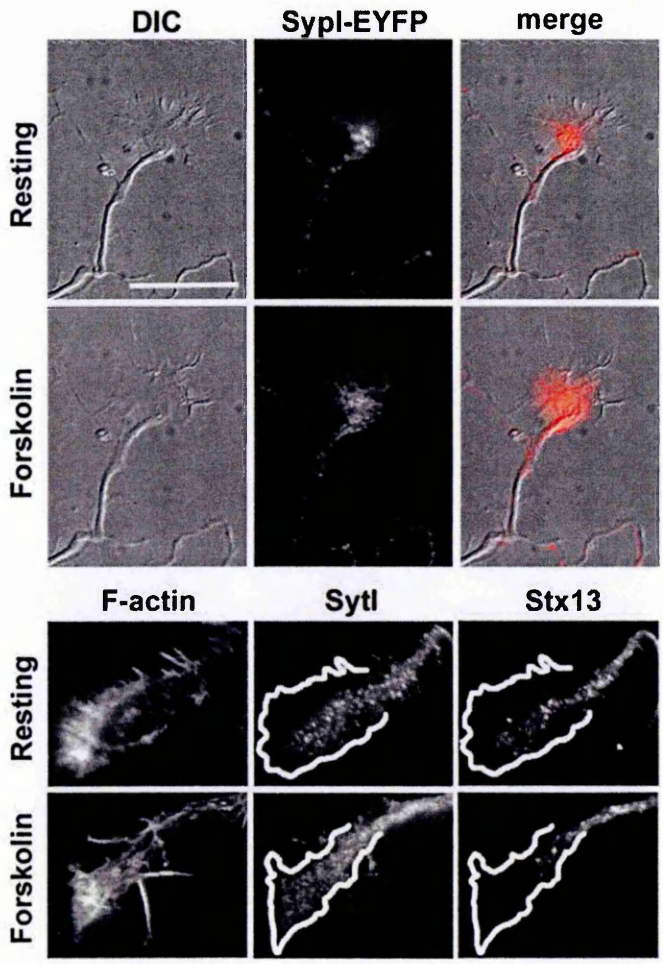

Figure 3.19. Elevation of intracellular cAMP leads to SV diffusion to the P domain. (Upper panel) Growth cone of a rat hippocampal neuron expressing SypI-EYFP, imaged before and after incubation with 50 μM forskolin for 10 min at RT. In the right column the overlay between the DIC and fluorescence (red) images is shown. At rest, SypI-positive vesicles are concentrated in the C domain, but they disperse throughout the growth cone after forskolin treatment. (Lower panel) Growth cones fixed either at rest or after treatment with forskolin for 5 min at 37° C, stained with antibodies against synaptotagmin I (SytI) and syntaxin 13 (Stx13), and with TRITC-conjugated phalloidin. The white trace outlines the distal edge of the P domain, as determined based on DIC images. Forskolin promotes the mobilization of synaptotagmin I-positive SVs to the P domain, whereas syntaxin 13-positive vesicles are still retained in the C domain. No

major change in the F-actin staining is visible upon forskolin treatment. Bar, 12 μm in the upper panel, 8 μm in the lower panel.

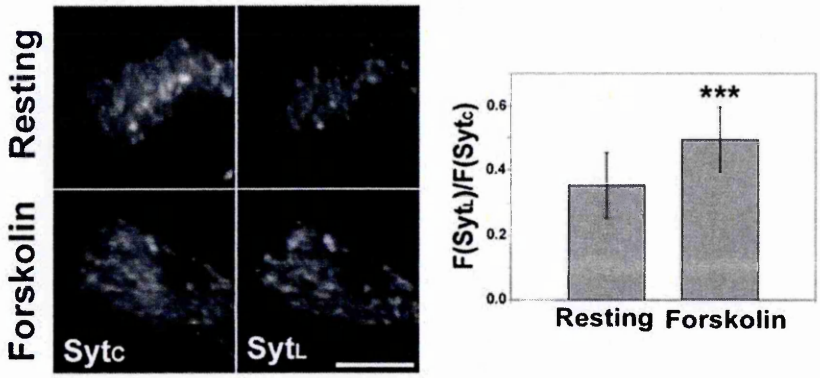

Figure 3.20. Elevation of intracellular cAMP enhances SV recycling in growth cones.
(Left panel) Growth cones of neurons incubated at 37° C with an antibody directed against the lumenal epitope of synaptotagmin I (Syt_L) for either 15 min in KRH or 5 min in 50 μM forskolin followed by 10 min in KRH. After incubation, neurons were fixed and counterstained with an antibody against total synaptotagmin I (Syt_C). (Right panel) Quantitative analysis of Syt_L internalization in growth cones either under control conditions or after treatment with forskolin. The ratio between the fluorescence intensities of Syt_L and Syt_C calculated for each growth cone is reported (mean ± SD; n=43). *** $p < 10^{-6}$ (Student's *t* test versus control growth cones). Bar, 5 μm.

3.2.4 Synapsins associate with recycling SVs and are phosphorylated by PKA in the growth cone

The SV-associated phosphoproteins of the synapsin family act as major regulators of the SV life cycle at the mature synapse by tuning the connection between SVs and F-actin in a phosphorylation-dependent manner also controlled by cAMP-dependent protein kinase (PKA) (De Camillli et al., 1990). Thus, we sought to investigate whether synapsins mediate the cAMP-dependent regulation of SV distribution and recycling in the growth cones. To identify and characterize the SV pool associated with synapsins in the growth cone, neurons were processed by double immunofluorescence for synaptotagmin I (to mark SVs) and total synapsin I. Bright fluorescent puncta of synapsin I immunoreactivity were associated with a fraction of the synaptotagmin I-

positive SVs localized in the C domain, although low levels of diffuse synapsin I immunoreactivity were also present in both the C and P domains of the growth cone (Figure 3.21 a-b''). A high degree of colocalization of synapsin I with synaptotagmin I was also detected at synaptic varicosities which at this developmental stage are only occasionally encountered in our cultures (arrowheads in Figure 3.21a).

The finding that synapsins preferentially associate with a subpopulation of SVs prompted us to determine whether these vesicles displayed distinct properties. Neurons were allowed to internalize the Syt_L antibody for 15 min in KRH and were subsequently counterstained for synapsin I. Remarkably, synapsin I showed an almost exclusive colocalization with constitutively recycling, i.e. Syt_L-positive, SVs (Figure 3.21 c-c'').

To investigate whether synapsin phosphorylation mediates the cAMP-dependent regulation of SV distribution and recycling in the growth cones, stage 3 neurons stimulated by either forskolin or K^+-induced depolarization were triple labeled with anti-synapsin antibodies which specifically recognize either phosphorylated site 1 (i.e. the PKA/CaMK I site), phosphorylated site 3 (i.e. one of the two CaMK II sites) or total synapsins. A parallel study of site 1 phosphorylation was carried out by Western blot analysis of total lysates of cultured neurons, followed by probing with site 1 phosphospecific anti-synapsin antibody (Figure 3.22). A basal level of site 1 phosphorylation was present at rest and was strongly reduced by H89, a PKA inhibitor. In the growth cones, enhancement of site 1 phosphorylation was induced by both forskolin and depolarization. BT-cAMP also promoted site 1 phosphorylation in total cell extracts. Consistent with previous reports, site 3 was virtually unphosphorylated under basal conditions and after treatment with forskolin, and was only weakly activated upon depolarization-induced Ca^{2+} influx in growth cones and developing axons (Menegon et al., 2002; Jovanovic et al., 2001). It is of interest to note that puncta of synapsin immunoreactivity were detected at rest, whereas after the application of

stimuli which enhance site 1 phosphorylation a diffuse pattern of synapsin fluorescence was present, possibly due to dissociation of synapsin from SVs upon phosphorylation (See below).

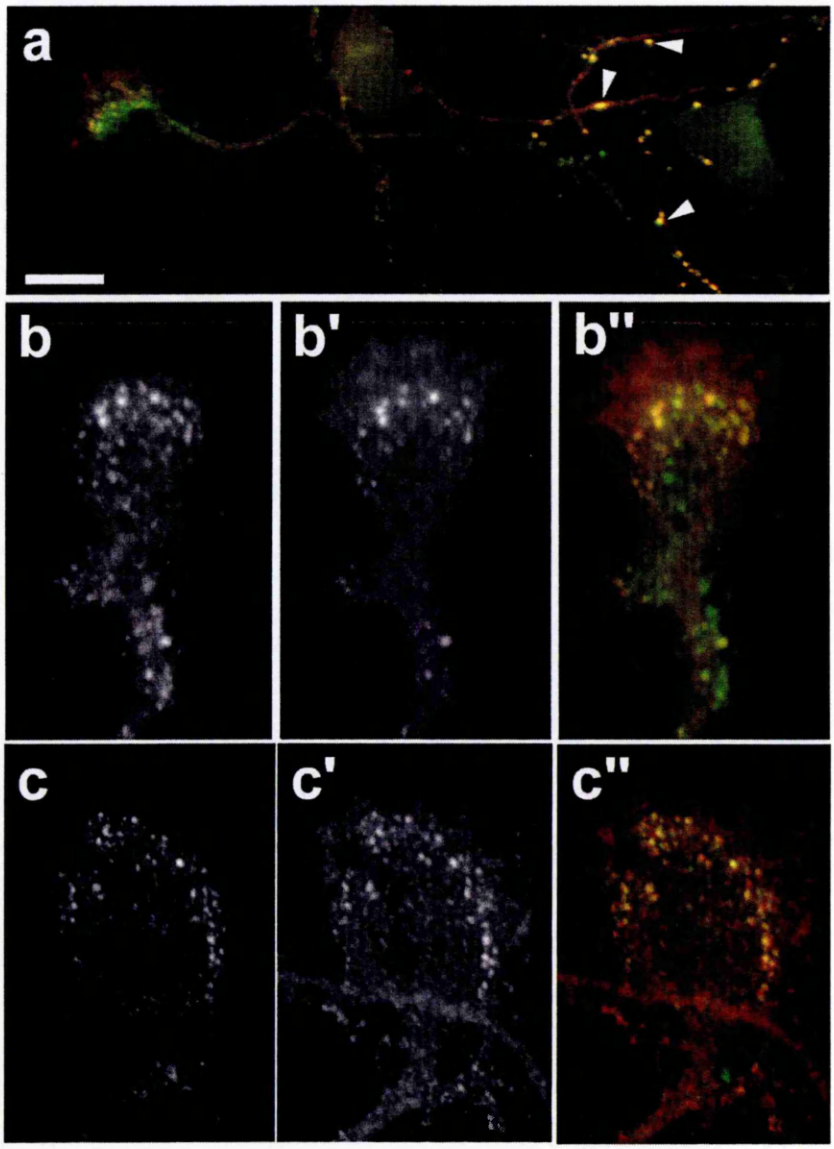

Figure 3.21. Synapsin associates with recycling SVs in growth cones.
(a-b'') Double immunofluorescence of rat hippocampal neurons stained with antibodies against synaptotagmin I (b; green in a and b'') and synapsin I (b'; red in a and b''). Synapsin I colocalizes with synaptotagmin I in a subpopulation of SVs in the growth cone and at virtually all synaptic varicosities along the axon (arrowheads in a). (c-c'') Neurons were incubated with the Syt_L antibody (c; green in c'') in KRH for 15 min at 37° C, fixed and counterstained with an antibody against synapsin I (c'; red in c''). Synapsin I associates with Syt_L-positive, i.e. recycling, SVs. Bar, 10 μm in a, 5 μm in b-c''.

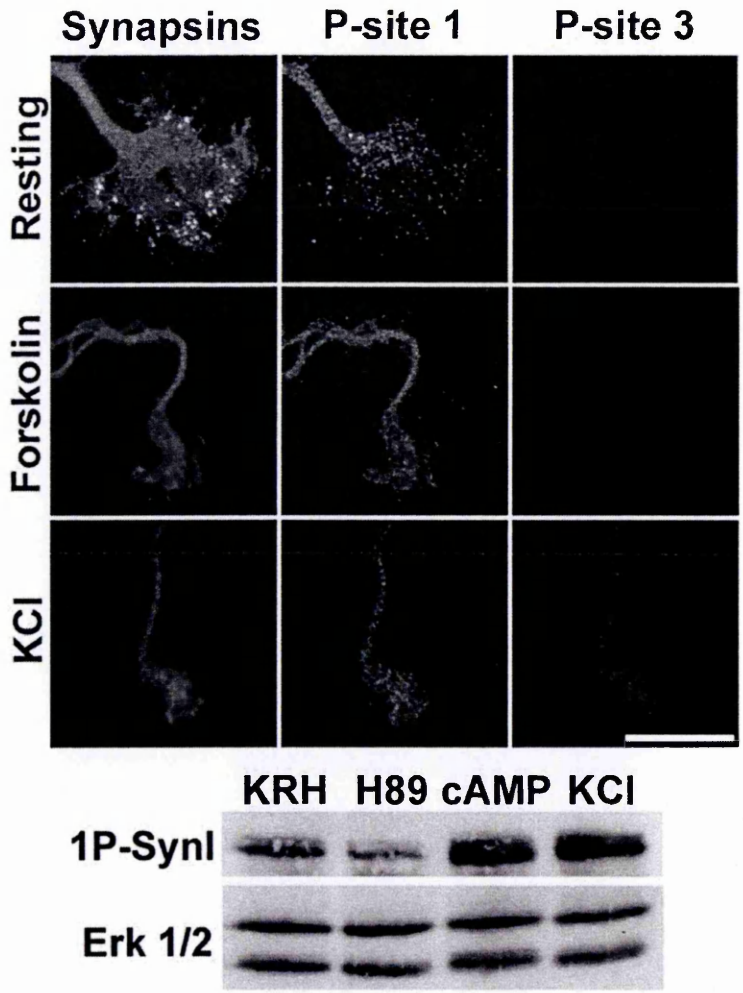

Figure 3.22. Synapsin is phosphorylated by PKA in growth cones.
(Upper panel) Growth cones of rat hippocampal neurons incubated at 37° C in the presence or absence of either 50 μM forskolin (for 10 min) or 55 mM KCl (for 1 min), fixed and processed for triple immunofluorescence with site 1 (P-site 1) and site 3 (P-site 3) phosphospecific anti-synapsin antibodies and with an antibody which recognizes total synapsins. At rest, a low level of basal site 1 phosphorylation is detectable. Phosphorylation at site 1 is enhanced by both forskolin and KCl, whereas site 3 is phosphorylated weakly and exclusively upon depolarization. Note that both treatments lead to the disappearance of the synapsin puncta visible in the control. Bar, 10 μm. (Lower panel) Cultured rat hippocampal neurons were lysed under control conditions (KRH) or after treatment with either 10 μM H89 for 30 min (H89), 10 mM BT-cAMP for 15 min (cAMP) or 55 mM KCl for 1 min (KCl). Equal amounts of protein were loaded into each lane. Parallel samples were probed with either a site 1 phosphospecific anti-synapsin antibody or an anti-ERK1/2 antibody. Basal site 1 phosphorylation is reduced by H89 treatment and enhanced by both BT-cAMP and depolarization.

3.2.5 Phosphorylation at site 1 controls the state of association of synapsin I with SVs in the growth cone

Synapsin phosphorylated at site 1 dissociates from SVs in vitro (Hosaka et al., 1999). To determine whether in the growth cones cAMP-dependent synapsin phosphorylation at site 1 is followed by dissociation from SVs, neurons incubated with forskolin were double stained for synaptotagmin I and synapsin I (Figure 3.23). At rest, synapsin colocalized with SVs in the C domain. After 3 min of treatment with forskolin, synapsin puncta were no longer detected, although synaptotagmin I-positive SVs were still clearly visible in the C domain and virtually absent from the P domain. In contrast, when the stimulation was prolonged for 10 min, mobilization of SVs into the P domain was also observed. This indicates that cAMP-induced synapsin dissociation from SVs precedes SV dispersion in the growth cone. At variance, synapsins remained associated with dispersed SVs after treatment with cytochalasin D (data not shown).

To unequivocally estimate the role of site 1 phosphorylation in the cAMP-induced dissociation of synapsins from SVs in the growth cones hippocampal neurons derived from mice lacking the synapsin I gene (Li et al., 1995) were co-infected with lentiviruses engineered to express SypI-EYFP and fusion proteins of ECFP with either wild type synapsin Ia (ECFP-SynI) or a mutant lacking phosphorylation site 1 (ECFP-SynI S9A) under the control of the PGK promoter. In order to provide a semi-quantitative evaluation of the colocalization between SypI-EYFP and the synapsin chimeras, we measured the relative fluorescence intensities along arbitrary lines in growth cones and preterminal axons. Under resting conditions, the intensity profiles of SypI-EYFP and of either synapsin chimera were highly superimposable, consistent with the idea that both wild-type and mutant synapsin I bind to SVs (Figure 3.24). After forskolin treatment, ECFP-SynI displayed a uniform distribution of fluorescence intensity with no correlation with the peaks of SypI-EYFP fluorescence. In contrast, the

intensity profiles of ECFP-SynI S9A fluorescence still matched those of SypI-EYFP, indicating that the state of association of synapsin I with SVs in the growth cone is entirely dependent on site 1 phosphorylation.

Together, these results indicate that changes in intracellular cAMP levels, which control SV distribution and recycling in the growth cone, also regulate the state of association of synapsin I with SVs.

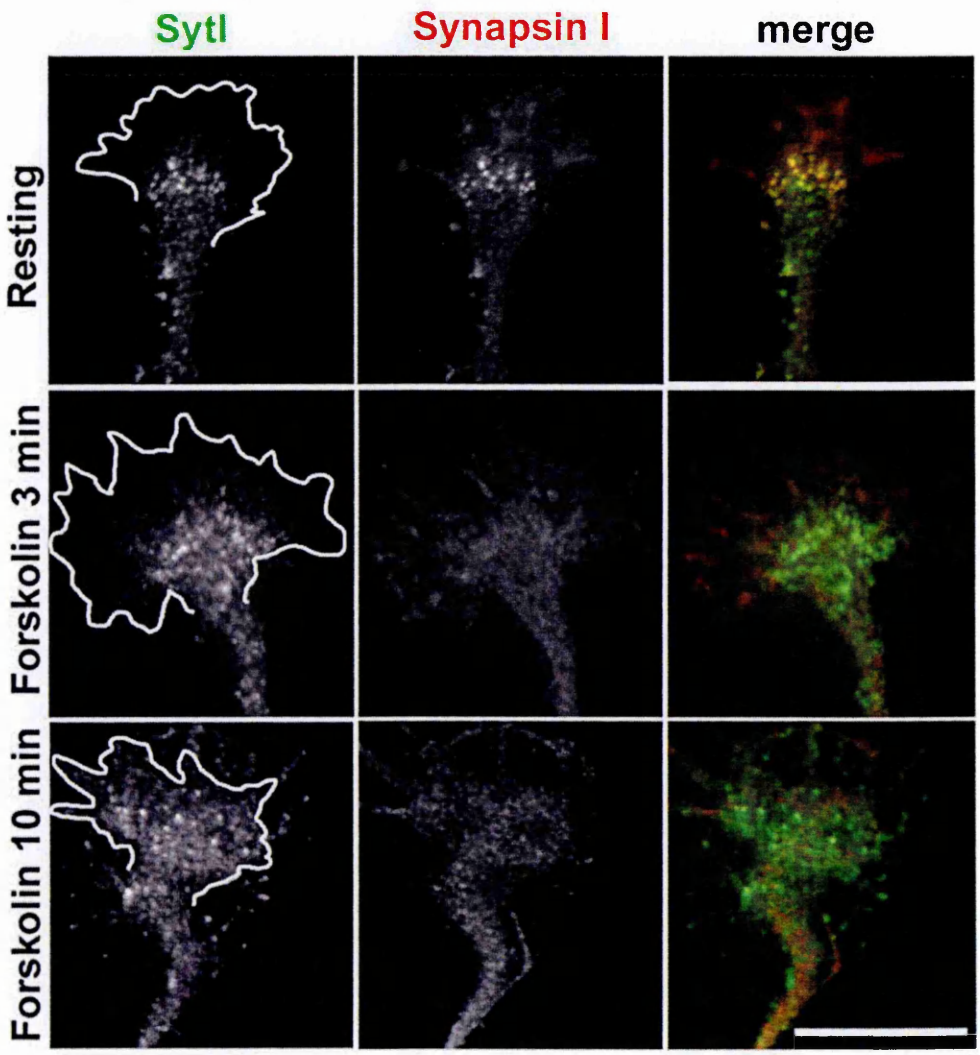

Figure 3.23. Dissociation of synapsin I from SVs precedes their diffusion to the P domain.

Growth cones of rat hippocampal neurons incubated for either 3 min or 10 min at 37° C in the presence or absence of forskolin, fixed and labeled with anti-synaptotagmin I (SytI; green in the merged images) and anti-synapsin I (red in the merged images) antibodies. At rest, synapsin I colocalizes with a fraction of synaptotagmin I-positive SVs, but after forskolin treatment it appears evenly distributed throughout the growth cone. Note that, 3 min after forskolin application SVs are still clustered in the C domain

whereas they become dispersed in the P domain when the stimulation is prolonged for 10 min. White traces in the left column outline the distal edges of the P domain, as determined based on DIC images. Bar, 10 μm.

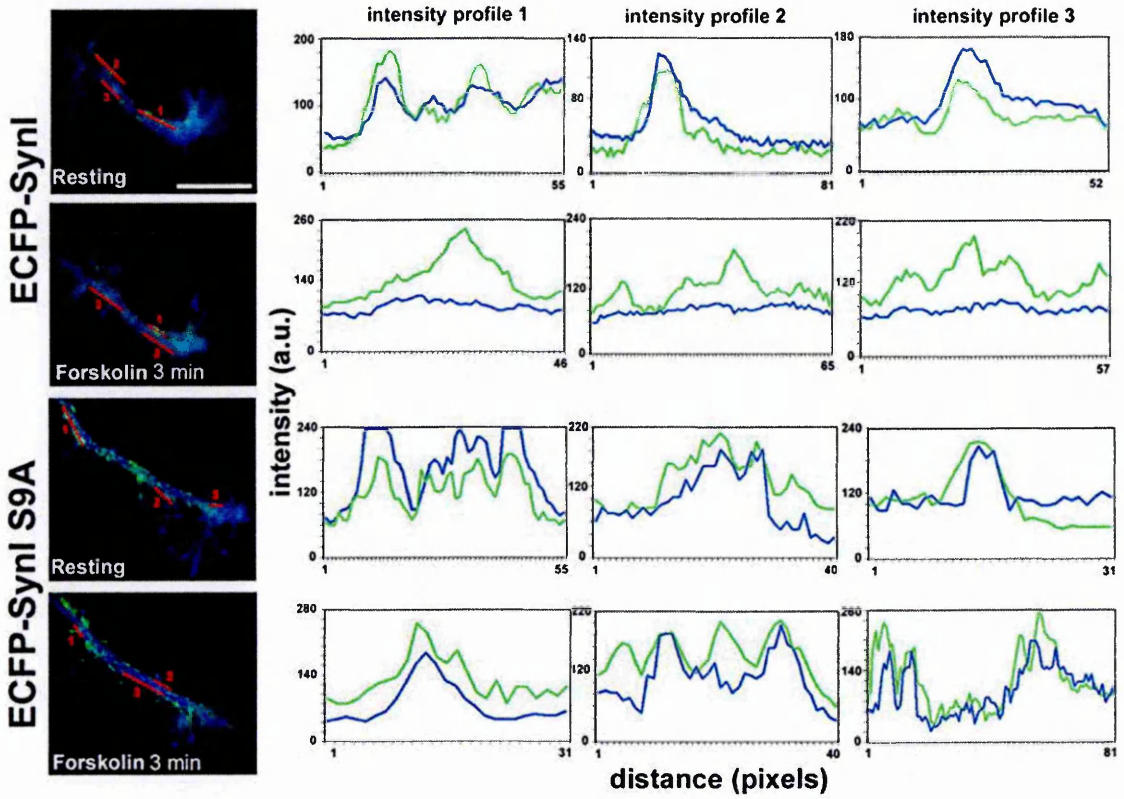

Figure 3.24. Phosphorylation at site 1 is required for the dissociation of synapsin I from SVs in the growth cone.
Growth cones and preterminal axons of hippocampal neurons from synapsin I knock-out mice double infected to express SypI-EYFP (green) and either ECFP-SynI or ECFP-SynI S9A (blue), imaged both before and after incubation with 50 μM forskolin for 3 min. The fluorescence intensity plots of SypI-EYFP (green traces) and of either ECFP-SynI or ECFP-SynI S9A (blue traces) measured along each of the numbered red lines are shown. Before treatment, the intensity peaks of both synapsin chimeras are largely superimposed with those of SypI-EYFP. After forskolin application, the ECFP-SynI S9A trace still peaks together with the SypI-EYFP trace, whereas the traces of ECFP-SynI and SypI-EYFP no longer overlap and ECFP-SynI becomes uniformly distributed. Note that the position of the lines has been modified after forskolin incubation because of the changes occurred over time in SV localization. Bar, 10 μm.

3.2.6 cAMP-modulated phosphorylation of synapsin I at site 1 controls the distribution of SVs in the C domain of the growth cone

I next investigated whether the changes in SV distribution and the dissociation of synapsin I from SVs in response to elevation of intracellular cAMP are causally related events. Firstly, SV distribution was altered in most growth cones of hippocampal neurons from synapsin I knockout mice (80%; n=70) (Figure 3.25). In the absence of synapsin I, synaptotagmin I-positive SVs did not concentrate in the C domain, but appeared to be distributed throughout the growth cone, reaching the distal edge of the P domain. A similar distribution was observed in the growth cones of neurons prepared from synapsin I/II/III triple knock-out mice (Figure 3.25), whereas in neurons prepared from synapsin II single knock-out mice SVs were clustered in the core of the growth cone, thus resembling the situation observed in wild-type animals (Figure 3.26A and data not shown).

Remarkably, expression of either ECFP-SynI or ECFP-SynI S9A restored the localization of SVs in the C domain of the growth cone of both synapsin I knock-out neurons (ECFP-SynI: 93%, n=45; ECFP-SynI S9A: 95%, n=45) and synapsin I/II/III triple knock-out neurons (ECFP-SynI: 89%, n=55; ECFP-SynI S9A: 94%, n=55) (Figure 3.25). Thus, both wild-type synapsin I and its mutant lacking phosphorylation site 1 promote clustering of SVs in the C domain of the growth cone under basal conditions. In addition, endogenous synapsin I was associated with synaptic vesicles in the growth cone of synapsin II-knock out neurons (Figure 3.26B). These findings rule out the need for synapsin I to form oligomers with the other synapsin isoforms in order to exert its effects on SVs distribution in the growth cone.

In order to study whether synapsin phosphorylation at site 1 plays a permissive role in SV dispersion induced by cAMP, neurons from synapsin I knock-out mice were double infected to express SypI-EYFP and either ECFP-SynI or ECFP-SynI S9A, and imaged

before and after incubation with forskolin. To allow a more accurate evaluation of the changes in SV distribution, the gray scale fluorescence images of SypI-EYFP were transformed into a pseudocolor spectrum, with warmer hues corresponding to higher fluorescence intensity. Interestingly, treatment with either forskolin or BT-cAMP led to dispersion of SVs in growth cones expressing wild type synapsin I, but was ineffective in those expressing the synapsin I S9A mutant (Figure 3.27 and data not shown). Thus, phosphorylation of synapsin I at site 1 is required in order to allow dynamic changes in SV distribution in response to intracellular cAMP increases.

In 2 growth cones expressing ECFP-SynI S9A out of 15 analyzed, we observed dispersion of SVs upon forskolin application (data not shown). Importantly, in both cases forskolin had induced an apparent alteration of the growth cone morphology, suggesting that a general enhancement of organelles movement can be achieved with more robust treatments, i.e. greater elevation of cAMP levels (see Forscher et al., 1987; Hollenbeck, 1993).

3.2.7 Phosphorylation of synapsin I at site 1 controls the rate of basal SV recycling in the growth cone

Levels of intracellular cAMP control basal recycling of SVs in growth cones. To test whether synapsin mediates the cAMP-dependent control of basal SV recycling, rat hippocampal neurons were infected with lentiviruses engineered to express either ECFP-SynI or ECFP-SynI S9A under the control of the CMV promoter, which allows to obtain high expression levels of the recombinant proteins. Uninfected neurons or neurons expressing ECFP-VAMP2 were used as controls. Basal SV recycling in these neurons was studied by quantitative analysis of Syt_L antibody uptake during a 15 min incubation in KRH. Expression of ECFP-SynI did not affect SV recycling. In contrast, constitutive SV recycling in ECFP-SynI S9A expressing growth cones was strongly

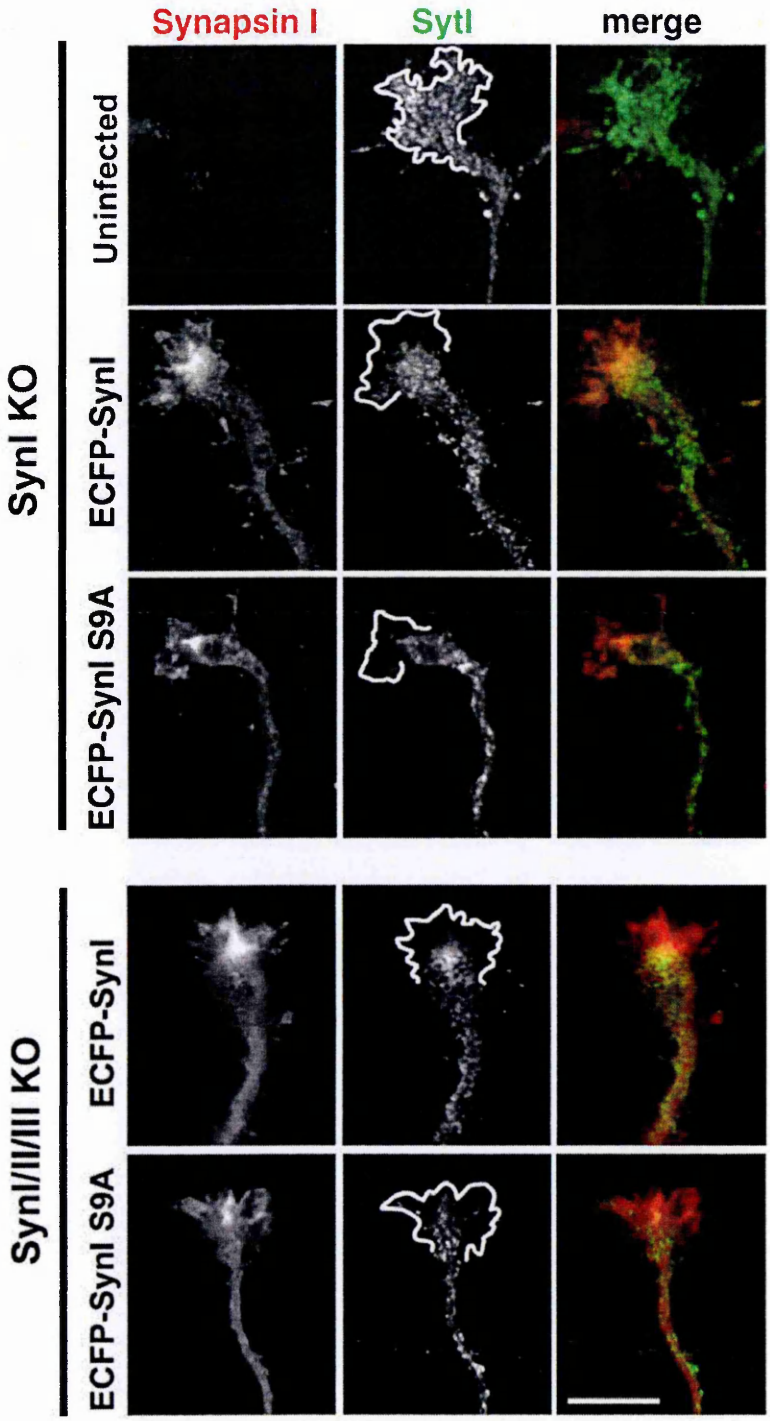

Figure 3.25. Synapsin I controls SV distribution in the C domain of the growth cone.

Hippocampal neurons from either synapsin I knock-out mice (upper panel) or synapsin I/II/III triple knock-out mice (lower panel) infected with either ECFP-SynI or ECFP-SynI S9A or left uninfected, fixed and stained with anti-synapsin (red in the merged images) and anti-synaptotagmin I (SytI; green in the merged images) antibodies. In the infected cells, the synapsin antibody recognizes both synapsin chimeras, while no synapsin immunoreactivity is detected in the uninfected sample. Synaptotagmin I-positive SVs are distributed throughout the growth cone in uninfected neurons, but confined to the C domain in both ECFP-SynI- and ECFP-SynI S9A-expressing growth cones. White traces in the middle column outline the distal edges of the P domain, as determined based on DIC images. Bar, 10 μm.

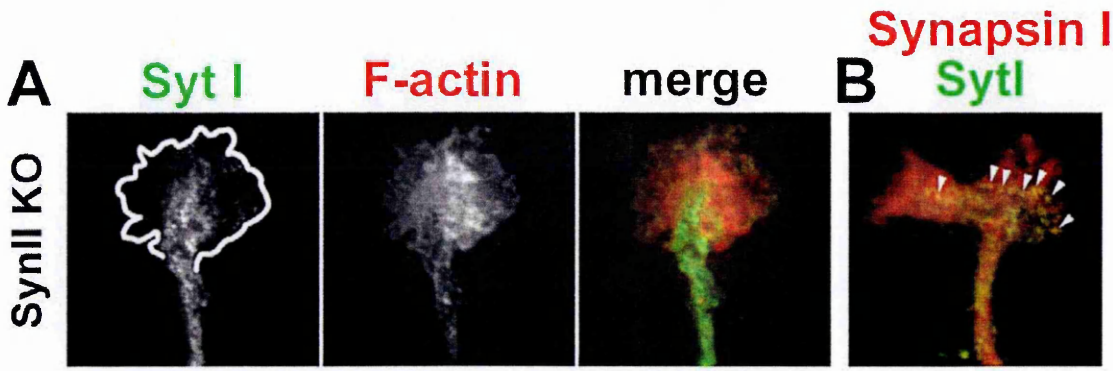

Figure 3.26. Synapsin II is dispensable for SV clustering in the C domain.
(A) Hippocampal neurons from synapsin II knock-out mice stained with an anti-synaptotagmin I (SytI; green in the merged image) antibody and TRITC-conjugated phalloidin (F-actin; red in the merged image). Synaptotagmin I-positive SVs are confined to the C domain in the growth cones of synapsin II knock-out neurons. White traces in the middle column outline the distal edges of the P domain, as determined based on DIC image. (B) Growth cone of a synapsin II knock-out neuron stained with anti-synaptotagmin I (SytI, green) and anti-synapsin I (red) antibodies. Synapsin I associates with synaptotagmin I-positive SVs even in the absence of synapsin II (arrowheads). Bar, 10 μm.

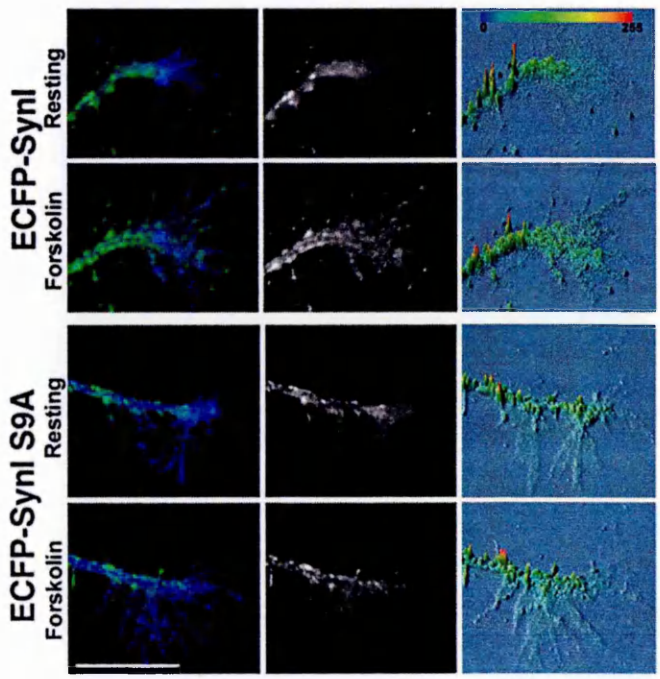

Figure 3.27. Site 1 phosphorylation of synapsin I is required for cAMP-modulated mobilization of SVs from the C domain of the growth cone.
Growth cones of hippocampal neurons derived from synapsin I knock-out mice co-expressing SypI-EYFP (green in the left column) and either ECFP-SynI or ECFP-SynI S9A (blue in the left column), imaged before and after incubation with 50 μM forskolin for 5 min at RT. The middle column shows SypI-EYFP fluorescence extracted from the merged images. In the right column SypI-EYFP image gray scales were transposed into a pseudocolor spectrum surface plot, with warmer hues corresponding to pixels of higher fluorescence intensity. Forskolin induces dispersion of SypI-EYFP-bearing SVs in growth cones expressing ECFP-SynI, but not in those expressing ECFP-SynI S9A. Bar, 10 μm.

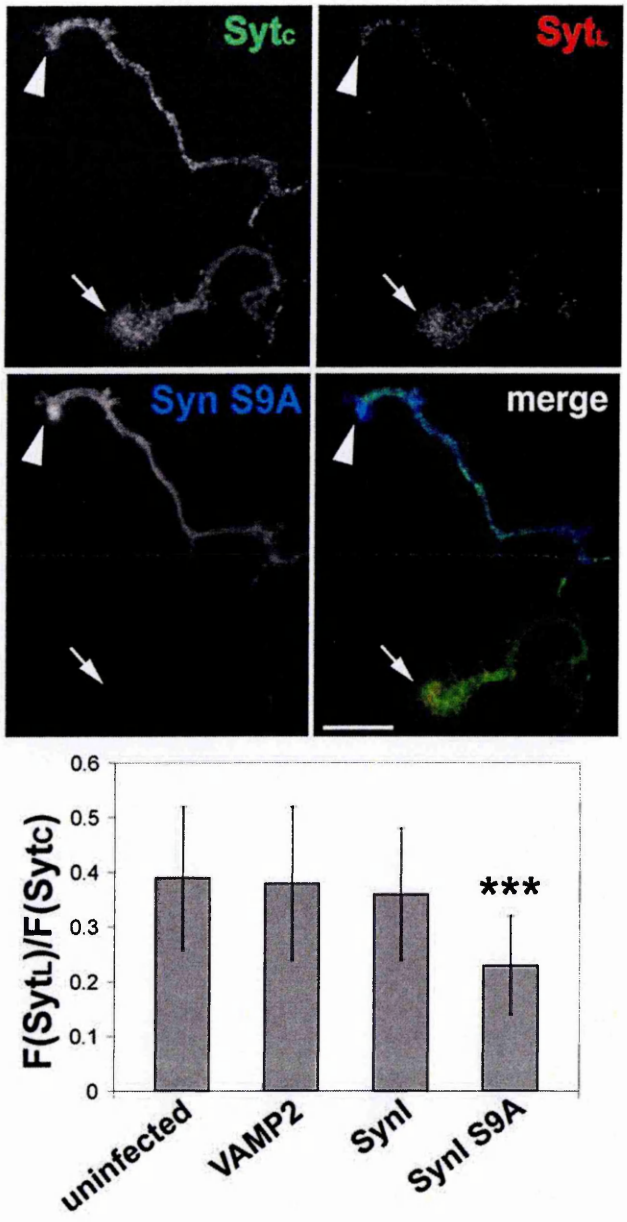

Figure 3.28. Site 1 phosphorylation of synapsin I controls the rate of SV recycling in the growth cone.

(Upper panel) Growth cones of rat hippocampal neurons infected with ECFP-SynI S9A (blue in the merged image) incubated with the Syt_L antibody (red in the merged image) in KRH for 15 min at 37° C, fixed and counterstained with an antibody against total synaptotagmin I (Syt_C; green in the merged image). The arrowheads and the arrows point to ECFP-SynI S9A-infected and uninfected growth cones, respectively. In spite of comparable levels of total synaptotagmin I, the ECFP-SynI S9A expressing growth cone displays a lower level of Syt_L internalization with respect to the uninfected growth cone. (Lower panel) Quantitative analysis of basal Syt_L internalization in growth cones expressing either ECFP-VAMP2, ECFP-SynI, or ECFP-SynI S9A. The ratio between the fluorescence intensities of Syt_L and Syt_C in each growth cone is reported (mean ± SD; n=50). The internalization of Syt_L antibody is reduced in the ECFP-SynI S9A-expressing growth cones (***p $<10^{-6}$, Student's *t* test, ECFP-SynI S9A versus uninfected growth cone). Bar, 10 μm.

reduced, when compared with recycling in either uninfected growth cones or growth cones expressing either ECFP-SynI or ECFP-VAMP2 (Figure 3.28). This indicates that synapsin I S9A, which lacks phosphorylation site 1, exerts a dominant negative effect on the basal rate of SV recycling in the growth cones.

3.2.8 Phosphorylation of synapsin I at site 1 controls synapse formation

To achieve long-term production of wild type or S9A mutant synapsin I in a time-window encompassing the peak of synaptogenesis in our culture system (Menegon et al, 2002), rat hippocampal neurons were infected at 1 day *in vitro* to express either ECFP-SynI or ECFP-SynI S9A under the control of the CMV promoter and were maintained in culture for 10-12 days. Remarkably, persistent ECFP-SynI expression strongly increased the density of synapses in the culture dish, whereas ECFP-SynI S9A induced a limited, not statistically significant, reduction in the number of synapses as compared to uninfected neurons (Figure 3.29A and B). In contrast, total neurite length measured on the basis of tubulin staining was unchanged by the expression of either synapsin chimera (data not shown).

We also quantified the intensity of VAMP2 immunoreactivity at single synaptic boutons to establish whether expression of either synapsin chimera promotes changes in the number of SVs per synapse. Expression of neither wild type nor S9A mutant synapsin I affected the levels of VAMP2-positive SVs (Figure 3.29C). Synapses positive for either ECFP-SynI or ECFP-SynI S9A were functionally active, since they displayed activity-induced SV exo-endocytosis as measured by FM 4-64 loading and unloading upon the application of depolarizing stimuli (data not shown).

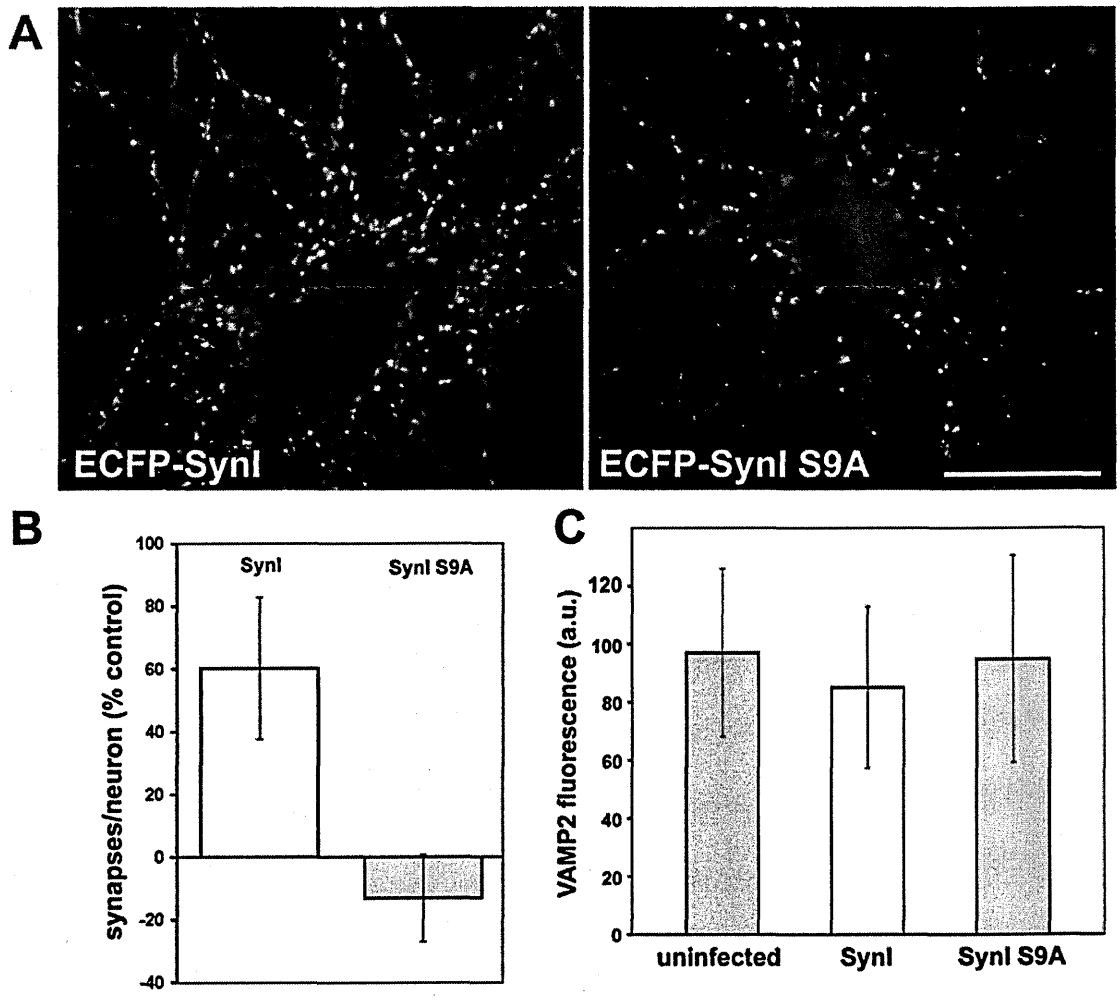

Figure 3.29. Long-term expression of synapsin I promotes synaptogenesis in a manner dependent on site 1 phosphorylation.
(a) Rat hippocampal neurons at 12 days *in vitro* expressing either ECFP-SynI or ECFP-SynI S9A under the control of a viral promoter were immunolabeled with an anti-GFP antibody followed by a goat anti-mouse IgG coupled to FITC. (b) Percent changes in the number of synaptic boutons per cell in neurons expressing either ECFP-SynI or ECFP-SynI S9A with respect to uninfected cells (mean ± SD from 4 independent experiments). (c) Levels of VAMP2 immunoreactivity in synapses from uninfected neurons and from neurons expressing either ECFP-SynI or ECFP-SynI S9A (mean ± SD; n=200). Bar, 10 μm.

DISCUSSION

4.1 MECHANISMS OF SYNAPTIC VESICLE PROTEIN SORTING

4.1.1 Sorting of SV proteins in neurons and non-neuronal cells

The molecular sorting events which lead to the formation of mature SVs are still poorly understood. In an attempt to clarify this issue, we took advantage of the use of spectrally separated variants of GFP fused in frame with various SV proteins to directly observe the fate of the resulting fluorescent chimeras in live hippocampal neurons.

The fluorescent chimeras of all the SV proteins tested were largely confined to the axon, indicating that they contain axonal targeting information. However, whereas synaptophysin I (SypI) was selectively confined to SVs, the other SV proteins, namely synaptotagmin I (SytI), VAMP2 and VAMP1 when overexpressed were not exclusively localized to synaptic sites. Rather, they were diffused all over the surface of the axonal plasma membrane, albeit some enrichment at the level of synaptic puncta could be detected. Thus, although these proteins bear the information to be sorted to the axon, they require additional signals to be recruited to SVs.

When ectopically expressed in cells that do not possess SVs, as Hela cells, VAMP2, SytI, and SypI show a differential subcellular localization. At steady state both VAMP2 and SytI are primarily associated with the plasma membrane, whereas SypI selectively accumulates in transferrin receptor-positive recycling endosomes (See also Cameron et al., 1991). However, a minor proportion of VAMP2 is also found associated with Rab5-positive early endosomes and transferrin receptor-positive recycling endosomes. At variance, SytI does not seem to be targeted to internal compartments and virtually all

the molecules accumulate on the cell surface (See also Feany et al., 1993; Jarousse and Kelly, 2001).

It should be considered that in neuron, membrane proteins are subjected to multiple sorting steps during their trafficking: they are sorted to the axonal or somatodendritic compartment and, within the same compartment, they may be incorporated into different organelles. According to studies carried out in neuroendocrine cells, it appears that SV proteins leave the TGN in vesicles of the constitutive secretory pathway. Indeed, in PC12 cells constitutive secretory vesicles mediate the transport of SypI from the TGN to the plasma membrane, in a process which involves fusion with endosomal compartments (Regnier-Vigouroux et al., 1991). Whether this pathway is taken also by other SLMV components and to what extent this model can be applied to neuronal SVs is unknown. However, it is becoming clear that any general model of protein targeting during SV biogenesis is likely to represent an oversimplification of the real situation and that each case has to addressed individually.

Studies concerning the SV protein VAMP2 have provided evidence for the existence of separate motifs for axonal sorting and for incorporation into SVs, raising the possibility that these are separate steps (Grote et al., 1995; West et al., 1997). Work from Banker's group indicates that accumulation of VAMP2 in the axon is due to retention, rather than to selective sorting, implying a role for molecular interactions which occur exclusively in this compartment (Sampo et al., 2003). Other studies have shown that when exogenously expressed in neurons VAMP2 displays a diffuse component of distribution along the axonal plasma membrane. This distribution was interpreted as either a result of protein spillover from synaptic sites upon SV exocytosis (Sankaranarayanan and Ryan, 2000; Li and Murthy, 2001) or lack of a developmentally regulated sorting control system (Ahmari *et al.*, 2000). In addition, mislocalization of VAMP2 to the axonal and dendritic plasma membrane has been reported in C. elegans mutants lacking

UNC-11, the homolog of the mammalian clathrin-adaptor protein AP180. Surprisingly, several other SV protein examined were normally distributed in the synaptic region in *unc-11* mutants (Nonet et al., 1999). The use of the VAMP2 mutant R56P, which cannot dissociate from the endogenous t-SNAREs after entering the SNARE complex, identifies the first target compartment of VAMP2, that is the first membrane acceptor at the level at which VAMP2 functions as a v-SNARE (Martinez-Arca et al., 2004). The expression of VAMP2 R56P in hippocampal neurons and Hela cells indicates that after leaving the TGN VAMP2 is not engaged in the formation of SNARE complexes until it reaches the plasma membrane. Interestingly, when expressed in neurons VAMP2 R56P localizes both to axonal and dendritic plasma membranes, showing that both domains are initial targets of VAMP2.

Thus, the picture emerging from these results indicates that VAMP2 is trafficked to the plasma membrane by carriers mediating the TGN to surface constitutive transport in both the axon and dendrites. After being incorporated into the plasma membrane, VAMP2 needs to establish a specific interaction with an adaptor complex in order to be sorted back to the axon and eventually to SVs. The sorting of VAMP2 from the plasma membrane to SVs may occur by either a direct endocytosis during SV re-formation or the targeting of VAMP2 into an endosome-mediated pathway devoted to the assembly of SV components into the same vesicle, or both. The involvement of an AP3-mediated step for VAMP2 sorting from endosomes to SLMVs has been reported in PC12 cells but not yet confirmed in neuronal cells (Salem et al., 1998).

However, it should be noted that in all these studies VAMP2 was transiently expressed in neurons under viral promoters, which typically produce high doses of the exogenous proteins. High expression levels might cause the missorting of the proteins from SV precursors budded from either the TGN or endosomes to constitutive secretory carriers.

I favor this interpretation to explain the accumulation of exogenous VAMP2 in the plasma membrane in both hippocampal neurons and Hela cells reported in this study. Indeed, when exocytosis is balanced by compensatory levels of endocytosis, SypI and other SV markers do not accumulate in the presynaptic plasma membrane (Valtorta *et al.*, 1988; Torri-Tarelli *et al.*, 1990 and 1992). However, endocytosis seems to be a saturable mechanism of SV retrieval which slows with stimulus increase, giving rise to the diffusion of SV proteins along the plasma membrane away from the sites of exocytosis (Sankaranarayanan and Ryan, 2000). SypI and VAMP2 have been shown to be recovered to SVs with similar kinetics after exocytosis, suggesting a similar mechanism of recovery for the two proteins during recycling (Li and Murthy, 2001). These findings suggest that the diffusion of some SV proteins along the axonal plasma membrane observed in this study might result from the saturation of the machinery involved in the recruitment of such proteins to SVs, and support a model in which SypI might play a pivotal role in directing VAMP2 targeting to SVs. Analogously, localization of VAMP2 and SytI to the plasma membrane in Hela cells is likely to be due to an overexpression artifact that saturated an essential component of the endocytotic machinery. However, the prominent surface localization of exogenous SytI in non-neuronal cells regardless the total expression level has been linked to the presence of a neuro-specific mechanism implicated in its internalization (Jarousse and Kelly, 2001).

In this study I confirm the selective targeting of SypI to transferrin receptor-positive endosomes reported by others (Johnston et al., 1989; Cameron et al., 1991; Linstedt and Kelly, 1991b). In addition, I show that, when expressed at high doses, SypI alters both the morphology and the recycling properties of transferrin receptor-positive endosomes: membrane recycling through the SypI-positive endosomes is reduced and these compartments appear enlarged. Moreover, in the enlarged endosomes SypI

accumulation is accompanied by a strong enrichment in cholesterol, as revealed by filipin staining. It is therefore possible that the presence of abnormally high levels of cholesterol in these endosomal membranes increases their stiffness, resulting in the impairment of vesicle budding from these compartments.

4.1.2 Dynamics of SynaptophysinI-VAMP2 interaction during SV exocytosis

VAMP2 is known to form a complex on the SV membrane with SypI (Calakos and Scheller, 1994; Washbourne *et al.*, 1995; Edelmann *et al.*, 1995; Galli *et al.*, 1996; Pennuto *et al.* 2002). Fluorescence resonance energy transfer (FRET) was measured in live neurons to study the dynamics of the SypI-VAMP2 complex during the exocytotic process. Taipoxin, a snake neurotoxin endowed with phospholipase A_2 activity (Kamenskaya and Thesleff, 1974; Fohlman et al., 1976), has been exploited to trigger SV exocytosis at synapses formed by cultured hippocampal neurons, using an experimental paradigm recently developed and validated (Pennuto et al., 2002; Rigoni et al., 2004).

Taipoxin, when applied in the presence of extracellular Ca^{2+}, induces massive SV exocytosis not followed by a proportionate SV membrane retrieval, resulting in the formation of bulges on axonal neurites. These bulges can be identified as swollen nerve terminals, since they correspond to areas where SV proteins are concentrated. At the doses of taipoxin employed in this study, swelling occurs only in a fraction of nerve terminals. Since the phospholipase A2 activity of the toxin has been shown to be Ca^{2+}-dependent, our findings that Tpx induces exocytosis and nerve terminal swelling exclusively in the presence of extracellular Ca^{2+} support a role for the enzymatic activity of the toxin in its mechanism of action.

The taipoxin-induced swelling of synaptic boutons was shown here to be accompanied by massive SV exocytosis, as indicated by the complete discharge of the lipophilic styryl dye FM4-64, which in contrast is retained in boutons that do not undergo swelling. This differential effect of the toxin on the synaptic terminals allows us to separately analyze the SypI-VAMP2 complex after the arrival of the fusogenic stimulus but either prior to SV exocytosis (the small boutons) or after SV exocytosis (the swollen boutons).

Remarkably, FRET analysis of the SypI/VAMP2 interactions in living neurons stimulated with Tpx at physiological $[Ca^{2+}]_{out}$ revealed that the two proteins interact on the SV membrane under resting conditions and dissociate after stimulation in both the normally sized and swollen synaptic boutons. This may be taken as an indication that the disruption of the SypI-VAMP2 interaction by Tpx precedes the enhancement of exocytotic fusion and the subsequent inhibition of endocytosis which lead to swelling of the terminals. These results are in accordance with the idea that SypI sequesters VAMP2 to prevent SNARE complex assembly in resting terminals. Release of VAMP2 is likely to occur in one of the steps that precedes fusion and make SVs competent for exocytosis upon Ca^{2+} influx (Figure 4.1B; See Pennuto et al., 2002).

Although these data, obtained in live neurons, do not formally prove that disruption of the SypI/VAMP2 interaction and enhancement of exocytosis induced by Tpx are mechanistically connected, they are in keeping with previous *in vitro* studies (Calakos and Scheller, 1994; Washbourne et al., 1995; Edelmann et al., 1995; Reisinger et al., 2004) supporting the modulatory role of the SypI/VAMP2 complex at synapses. Release of VAMP2 from SypI appears to precede fusion and might be a prerequisite to make SVs competent for exocytosis (See also Reisinger et al., 2004). However, disruption of the SypI/VAMP2 interaction by Tpx is not sufficient per se to promote SV

exocytosis, since it was also observed in those terminals in which SV had not undergone massive fusion, i.e the small synaptic boutons.

These results expand the previous findings reported by Pennuto et al. (2002) obtained using α-latrotoxin to elicit SV fusion. Indeed, at variance with α-latrotoxin, taipoxin induces exhaustive SV exocytosis exclusively when applied in the presence of extracellular Ca^{2+}, thus permitting the investigation of the dynamics of the SypI/VAMP2 interactions at physiological Ca^{2+} levels.

4.1.3 Synaptophysin I governs the sorting of VAMP2 in neurons and non-neuronal cells

A possible function of SypI in SV biogenesis has been hypothesized based on its ability to interact with cholesterol (Thiele et al., 2000). SypI might be involved in the formation of lipid microdomains where SV membrane constituents are pre-assembled, a situation similar to that observed for the apical transport of proteins in epithelial cells. In addition, the ability of SypI to form oligomers might promote membrane curvature, facilitating the budding of SVs from the donor membrane (Hannah et al., 1999). Thus, SypI appeared to be a potential candidate for rescuing the correct targeting of the other SV proteins.

Interestingly, SypI was selectively able to recruit axonal VAMP2 to SVs. The localization of both proteins on functional SVs was indicated by their colocalization with endogenous SV markers and by the redistribution of the fluorescent signal to the presynaptic plasma membrane upon α-latrotoxin-stimulated exocytosis (Pennuto et al., 2002). The effect of SypI on VAMP2 was dose-dependent, and correct targeting of VAMP2 to SVs was achieved when the range of expression of the exogenous proteins was similar to that of the endogenous proteins. On the other hand, neither VAMP1 nor SytI could be recruited to SVs even at very high SypI expression levels.

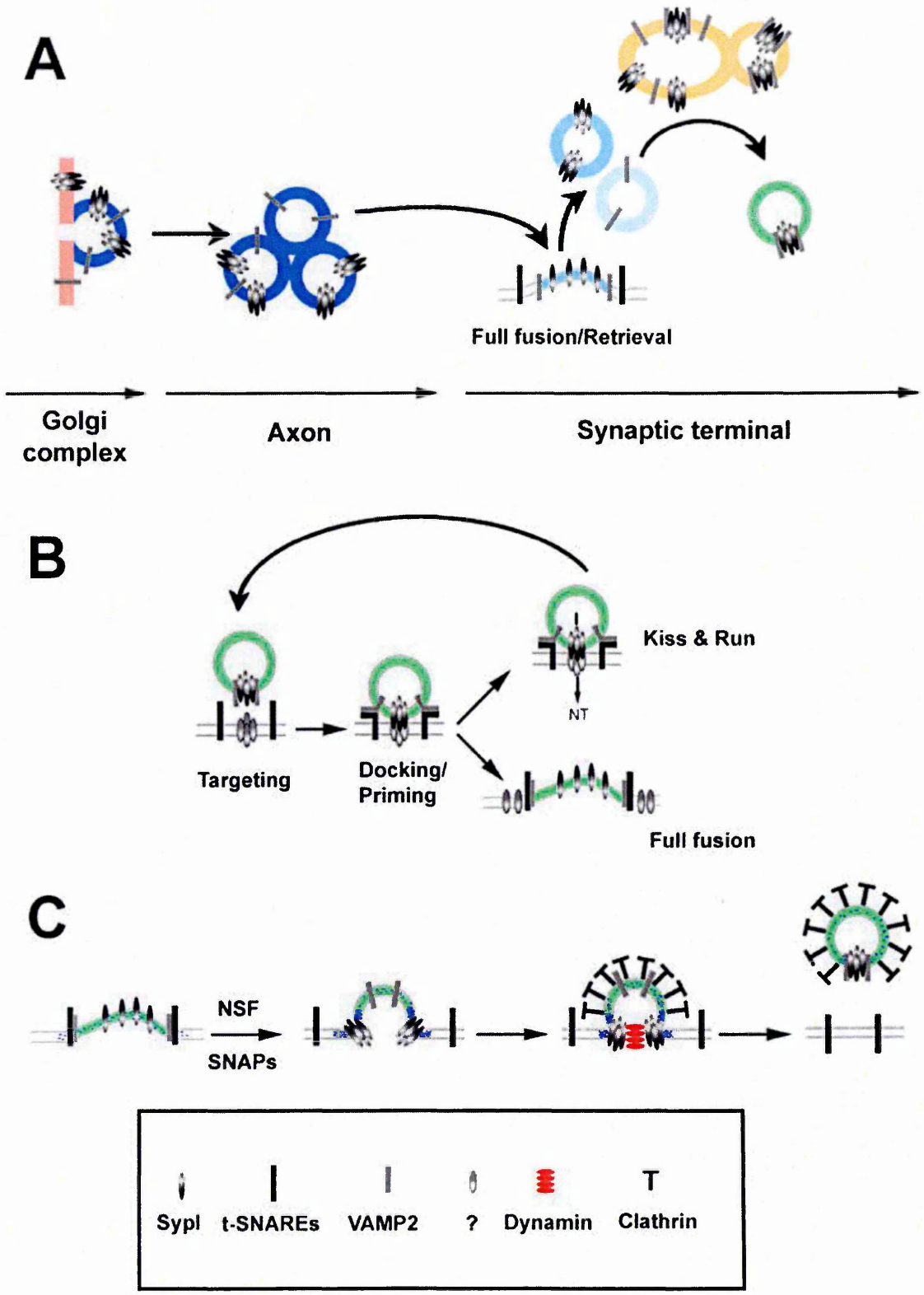

Figure 4.1. Possible roles of Syp in SV exo-endocytosis.
(A) At the level of the Golgi complex Syp might play a role in the assembly of specialized domains enriched in SV components on the Golgi membrane. Syp oligomers are sorted onto SV precursors, possibly together with other SV proteins, including VAMP2. The Syp/VAMP2 interaction might take place already at this stage,

and be crucial for the direct sorting of VAMP2 to SV precursors (i.e. transport packets), and hence for VAMP2 targeting to synaptic sites. Alternatively, the generation of SVs in which Syp oligomers interact with VAMP2 might occur only at the synaptic terminal and require a passage through an endosomal compartment after fusion and retrieval of trasport packets.
(B)The Syp/VAMP2 interaction is lost prior to full fusion of SVs with the pre-synaptic plasma membrane and dissociation of Syp oligomers. When the SV is docked at the plasma membrane, Syp oligomers might be put in register with an as yet unknown plasma membrane component, allowing the formation of a channel for rapid neurotransmitter release during the kiss-and-run mode of SV exocytosis.
(C) Syp might participate in clathrin-dependent SV endocytosis from the pre-synaptic plasma membrane. Shifting of Syp from the monomeric to the oligomeric state might be accompanied by enrichment of cholesterol (blue dots) in specific membrane domains, thus fostering the generation of the negative membrane curvature needed for SV budding. The fission step might be facilitated by the interaction of Syp with dynamin.

The fact that SypI is unable to redirect the sorting of VAMP1, although the latter is highly homologous to VAMP2, suggests that the effect of SypI on VAMP2 sorting requires a direct interaction between the two proteins. Indeed, at mature synapses SypI is known to interact with VAMP2, whereas the possibility of a direct interaction with VAMP1 is controversial (Calakos and Scheller, 1994; Edelmann et al., 1995; Washbourne et al., 1995; Bacci et al., 2001). As far as SytI is concerned, the existence of a direct interaction between SytI and SypI on the SV membrane has been excluded (Pennuto et al., 2002), although the two proteins have been shown to be transported along the axon in the same carrier vesicle (Okada et al., 1995).

Overall, VAMP1 and VAMP2 sequences show a high degree of similarity. Interestingly, they maximally diverge in their proline-rich amino-terminal portions, which in the case of VAMP2 has been shown to be required for its interaction with SypI (Washbourne et al., 1995; Bacci et al., 2001). Consistently, when the amino-terminal region of VAMP2 was substituted with that of VAMP1, the resultant protein diffused irrespective of the presence of SypI. Vice versa, when the amino-terminal region of VAMP1 was replaced by that of VAMP2, the chimera was seen to diffuse when expressed in the absence of SypI, whereas it concentrated at synaptic sites when

expressed in the presence of SypI. These findings indicate that the cytosolic tail of VAMP2, which is responsible for its interaction with SypI, is also necessary for its SypI-mediated recruitment to SVs. It has previously been reported that the cytosolic tail of VAMP2 contains a sequence which negatively regulates its targeting to SVs (West et al., 1997). Our results are consistent with the hypothesis that SypI, by interacting with VAMP2, leads to the masking of this negative regulator, thus allowing the retention of VAMP2 in SVs.

In the PC12 neuroendocrine cell line, SypI has been shown to exit the TGN in constitutive vesicles, and to undergo at least one cycle of fusion with the plasma membrane and recycling through endosomes before being incorporated into SLMVs (Regnier-Vigouroux et al., 1991). If this behaviour can be extrapolated to neurons, then it is possible that the interaction of SypI with VAMP2 at some step of the recycling process leads to the recruitment of the latter to SVs.

Two possibilities must be taken into account: SypI might specifically recruit newly synthesized VAMP2 (maybe together with other SV components) at the level of the TGN or might recruit SV components directly at the nerve terminal, from either the plasma membrane or endosomes (Figure 4.1). At the plasma membrane SypI could organize lateral membrane domains enriched in cholesterol and enhance the endocytotic process involved in SV formation (Martin, 2000). Through a hierarchy of protein-protein interactions, some of which directly involve SypI, a complement of SV components might accumulate at these raft-like elements, preassembled ready for the rapid endocytic regeneration of the SV, which might be favored by the interaction of SypI with dynamin. In addition, clathrin-dependent endocytosis of the SV-raft is expected to be further enhanced by the enrichment in proteins such as VAMP2 and SytI, that possess cytoplasmic endocytic motifs and interact with adaptor complexes (See Hannah et al., 1999 for references).

This study does not support a generalized role for SypI in directing the sorting of SV proteins. Thus, if SypI organizes cholesterol-rich microdomains at the plasma membrane where SV proteins selectively accumulates, these raft-like elements are probably not sufficient to mediate the targeting of all SV components, and additional factors are likely to be involved. Interestingly, in Syp knock out animals a reduction in the levels of VAMP2, but not of other SV components, has been observed (McMahon et al., 1996).

SV formation from PC12 endosomes *in vitro* is inhibited by the incubation of the donor membrane with an antibody directed to VAMP2, whereas no inhibition is detected using an antibody against SypI (Salem et al., 1998). This result indicates that SypI is not required for the generation of SVs from an endosomal compartment via AP3 complex recruitment.

In addition, it has recently been reported that VAMP2 is delivered to both axons and dendrites, but is preferentially endocytosed from the dendritic membrane. Thus, its polarized distribution to the axonal compartment can be ascribed to selective retention, rather than to selective delivery (Sampo *et al.*, 2003). SypI is unlikely to be involved in mediating the selective retention of VAMP2 in the axonal compartment, since the latter accumulates in the axonal membrane also when overexpressed in the absence of stoichiometric amounts of SypI. However, the SypI-VAMP2 interaction appears to be necessary for recruiting VAMP2 to SVs, either by inducing endocytosis of VAMP2 from the axonal plasma membrane or by facilitating sorting from the endosomal compartment (Figure 4.1).

The expression of SV proteins in Hela cells permits further analysis of the mechanism underlying the effect of SypI on VAMP2 sorting. First, I show that SypI exerts a selective effect on VAMP2 sorting in non-neuronal cells, causing the redistribution of exogenous VAMP2 from the plasma membrane to intracellular compartments. At

variance, exogenous SytI retains a prominent plasma membrane targeting when co-expressed with SypI. Importantly, the formation of the VAMP2-SypI complex was detected biochemically in Hela cells. The remarkable analogy between the situation observed in neurons and in non-neuronal cells indicates that the ability of SypI to selectively control the sorting of VAMP2 does not require a functional context (i.e., the SV membrane).

The effect of SypI on VAMP2 sorting in Hela cells is not reproduced by overexpression of Rab11 which, similarly to SypI, causes the enlargement of recycling endosomes and promotes the accumulation of cholesterol in these compartments. Thus, the effect of SypI on protein sorting is specific for VAMP2 and is not due to a general impairment of protein trafficking through recycling compartments. A specific factor is likely to be required for SV generation from the endosomal compartments where SypI and VAMP2 are co-targeted. It is noteworthy that the overexpression of either the four-transmembrane domain SV protein synaptogyrin or its non-neuronal paralog cellugyrin, distantly related to SypI , dramatically increases the SypI content of SLMVs in PC12 cells (Belfort and Kandror, 2003). In addition, cellugyrin promotes the biogenesis of SLMVs, thus suggesting that the mechanism for incorporating SypI into the vesicles relies on efficient cellugyrin-dependent SV generation (Belfort et al., 2005). Although synaptogyrin and cellugyrin are efficiently targeted to SLMVs in PC12 cells or specialized microvesicles in other cell types (Belfort and Kandror, 2003), it remains to be investigated whether, en route to their final destination, they are trafficked through endosomal compartments, where they might recruit SypI and, in turn, VAMP2, to the vesicle.

A major result obtained in Hela cells is to show that endocytosis is not required for the SypI-directed sorting of exogenous VAMP2, at least in non-neuronal cells. This observation implies that the VAMP2 sorting step influenced by SypI occurs at either the

TGN membranes or endosomal compartments where the two proteins are targeted directly from the TGN without recycling through the plasma membrane. However, the evidence that the plasma membrane is the first target of VAMP2 (Martinez-Arca et al., 2004), strongly suggests that SypI and VAMP2 are sorted together at the level of the Golgi membranes. Whether a direct interaction between the two proteins is required for directing their coordinated exit from the TGN remains to be determined, although the data obtained in neurons suggest that this is the case. It should be noted anyway that the only data available in the literature argue against the sorting of SypI and VAMP on the same vesicle budded from the TGN. Indeed, in the perikarion VAMP2 does not co-distribute with SypI in TGN-derived tubules induced by BFA (Mundigl et al., 1993).

The model that I propose, according to which SypI and VAMP2 interact in the TGN and exit together directed to the plasma membrane, highlights an essential function for SypI in escorting VAMP2 to the sites where exocytosis must take place exclusively after the arrival of the appropriate stimulus, which causes dissociation of the SypI-VAMP2 complex, thus leaving VAMP2 available to participate in the formation of the SNARE complex. This mechanism ensures that SV precursors endowed with the v-SNARE VAMP2 are kept unable to fuse until they reach the correct destination.

Interestingly, the long carboxy-terminal domain of SypI, which is required for efficient regulation of VAMP2 sorting, is dispensable for the SypI/VAMP2 interaction as well as for the formation of SypI homo-oligomers (Pennuto et al., 2002; this study). Since this region contains an endocytic motif and several potential tyrosine-based phosphorylation sites, this result might indicate that the control of VAMP2 sorting and function by SypI, which relies on the direct interaction between the two proteins, needs additional regulatory processes, such as post-traslational modifications and association with other proteins.

The apparent discrepancy between the results presented in this study and the lack of phenotype in mice deleted for the *sypI* gene (Eshkind and Leube, 1995; McMahon et al., 1996) might be due to compensatory effects exerted by other members of the synaptophysin family (see, e.g., Spiwoks-Becker et al., 2001; Janz et al., 1999). Indeed, a role for SypI in activity-dependent synapse formation has been highlighted by the use of heterogenotypic cocultures of neurons from wild type and knock-out mice (Tarsa and Goda, 2002). Thus, when synaptogenesis occurs under competitive conditions, such as for example in Xenopus neuronal cultures in which only half of the cells had the Syp levels manipulated by the injection of either anti-Syp antibodies (Alder et al., 1992) or Syp mRNA (Alder et al., 1995), an important functional role of Syp in synaptic physiology becomes apparent.

4.2 SYNAPTIC VESICLE DYNAMICS IN DEVELOPING NEURONS

4.2.1 Synapsin I controls SV distribution and recycling in the growth cones

In this study I show that SV distribution and recycling in hippocampal neurons developing in culture are already subjected to regulatory mechanisms similar to those operating at the mature synapse. cAMP-dependent pathways control the spatial organization and basal rate of recycling of SVs in the growth cones and both effects require phosphorylation of synapsin I at a conserved PKA/CaMKI site. cAMP-stimulated phosphorylation of synapsin I controls its association with SVs in the growth cone. The lack of expression of synapsin I impairs the ability of the growth cone to retain SVs in the C domain under basal conditions. Reintroduction of synapsin I restores the correct localization of SVs. This effect does not require the formation of heterodimers between synapsin I and the other synapsin isoforms (Hosaka and Sudhof, 1999), since it can be reproduced by heterologous synapsin I expressed in synapsin I/II/III triple knock-out neurons. In addition, changes in SV distribution in response to intracellular cAMP increase and constitutive SV recycling in the growth cone of isolated axons are impaired in neurons expressing a mutated synapsin I lacking the PKA phosphorylation site.

The model emerging from these results indicates that synapsin I, by tethering SVs to the F-actin meshwork enriched at the C-P domain interface, maintains the distribution of SVs in the C domain of the growth cone. Phosphorylation of synapsin I at site 1 leads to its dissociation from SVs, which are thus available for exocytosis. Therefore, any change in cAMP levels which leads to PKA activation and synapsin phosphorylation can modulate SV availability for recycling (Figure 4.2).

Thus, it appears that the same molecular mechanisms which are at the basis of the role of synapsin in modulating neurotransmitter release from mature nerve terminals underlie also the role of the protein in SV dynamics in growth cones. Indeed, synapsin phosphorylation is regulated by synaptic activity (Nestler and Greengard, 1982; Sihra et al., 1989) and is associated with profound changes in its affinity for SVs and actin (Valtorta et al., 1992; Benfenati et al., 1989; Hosaka et al., 1999). Consistently, phosphorylated synapsin I dissociates from SVs and diffuses in the axon during high-frequency stimulation (Torri Tarelli et al., 1992; Chi et al., 2001). Thus, in mature nerve terminals synapsin phosphorylation regulates the tethering of SVs to the actin cytoskeleton, leading to changes in the availability of SVs for exocytosis. Similarly, in growth cones PKA-mediated phosphorylation of synapsin I regulates the spatial organization of SVs and their ability to undergo exo-endocytosis.

cAMP-dependent pathways play an important role in the regulation of neurotransmitter release at the mature synapse (Trudeau et al., 1996; Munno et al., 2003). The relevance of cAMP-dependent control of SV recycling during development has been illustrated by experiments showing that spontaneous release of acetylcholine from growth cones of cultured Drosophila central neurons is impaired in fly memory mutants with constitutively enhanced PKA activity (Yao et al., 2000).

In addition to synapsins, which are the most abundant substrate for PKA in adult neurons (Johnson et al., 1972), other synaptic targets of PKA can also be involved in the regulation of neurotransmitter secretion in developing neurons. Other presynaptic targets of PKA include α-SNAP (Hirling and Scheller, 1996), Ca^{2+} channels (Leveque et al., 1994), Cystein String Protein (Graham and Burgoyne, 2000), Snapin (Thakur et al., 2004), SNAP-25 (Nagy et al., 2004), RIM1α (Lonart et al., 2003). Although we cannot rule out the possibility that other presynaptic proteins participate in the cAMP-

mediated control of SV dynamics in the growth cone, phosphorylation of synapsin I by PKA appears to play a major role in this phenomenon.

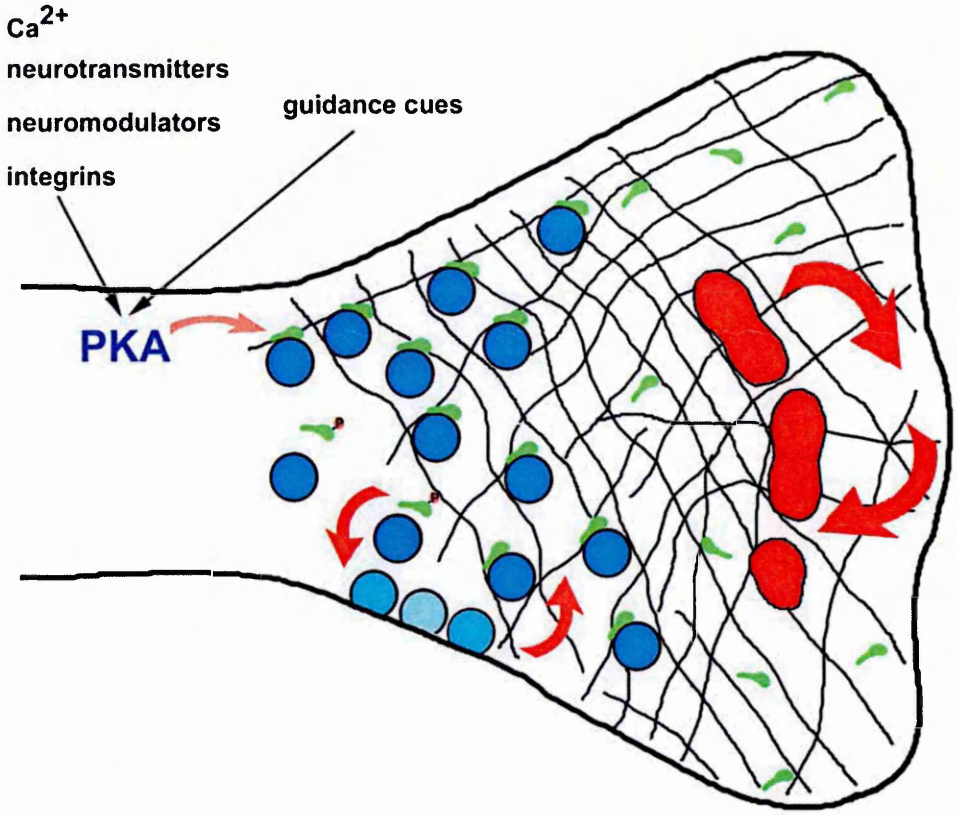

Figure 4.2. Synapsin I controls the distribution and recycling of SVs in the growth cone.
In the growth cone synapsin I (green) tethers SVs to F-actin, thus maintaining SVs clustered at the interface between the C and P domains where F-actin is highly enriched. Multiple extracellular signals, including adhesion molecules and guidance cues, as well as changes in $[Ca^{2+}]_{in}$, modulate cAMP production and hence PKA activity. Upon phosphorylation by PKA, synapsin I detaches from SVs which become available for mobilization and exo-endocytosis. The control exerted by synapsin I on SV dynamics prevents SVs to be engaged in the rapid membrane recycling processes associated with the transition domain of growth cone (red vacuoles).

cAMP is known to promote Ca^{2+} entry in the presynaptic terminal at the neuromuscular junction (Funte and Haydon, 1993). However, it is unlikely that the observed effects are due to cAMP-induced activation of Ca^{2+}/calmodulin dependent kinases (CaMKs).

Indeed, synapsin phosphorylation by CaMK II at sites 2/3 is undetectable in developing axons (Figure 3.22; Menegon et al., 2002) and studies with kinase inhibitors indicate that CaMK I has a limited role in the control of synapsin function mediated by site 1 phosphorylation (A. Menegon, D. Bonanomi, F. Valtorta, unpublished observations). Whether in developing neurons neurotransmitter release is regulated at multiple levels by different signaling pathway, as occurs at the mature synapse, remains to be established. Interestingly, PKC activation leads to a substantial increase in spontaneous neurotransmitter release from the growth cone, indicating that other signaling cascades can cooperate with cAMP-dependent pathways to control SV life cycle during development (Girod et al., 1995; Gao and van den Pol, 2000). Intriguingly, synapsin I is phosphorylated at both the PKA and CaMK II sites upon PKC activation in the hippocampus (Browning and Dudeck, 1992).

4.2.2 The competence of SVs to undergo recycling is regulated prior to synapse formation

The axonal growth cone is a site of intense membrane trafficking (Diefenbach et al., 1999). However, thus far the possibility that the trafficking of the various organelles present in this compartment is differentially regulated had not been addressed. Using the lipophilic dye FM 4-64, I have visualized an intense process of membrane recycling characterized by rapid kinetics. However, SVs do not appear to be engaged in such fast recycling, and their exo-endocytosis occurs at a much slower rate, which can be visualized by uptake of the antibody directed against the luminal domain of synaptotagmin I. The relative stillness of SVs, which appear to be retained in the central core of the growth cone, is suggestive of the existence of a control device operating prior to the formation of synaptic contacts to limit SV recycling.

The presence of an inhibitory mechanism to limit spontaneous neurotransmitter release from isolated axons prior to the contact with the target cell has been shown previously in Xenopus spinal neurons (Xie and Poo, 1986; Sun and Poo, 1987). Spontaneous release is inhibited in isolated neurons and this inhibition is reduced upon contact with the post-synaptic cell.

Thus, the basal rate of SV exocytosis, which is likely to mediate the process of neurotransmitter release and hence neuronal communication already at these early stages, is dissociated from the general process of constitutive exocytosis of plasma membrane precursor vesicles and other ill-defined organelles which leads to plasma membrane expansion and neurite outgrowth (Lockerbie et al., 1991; Leoni et al., 1999). Consistently, constitutive secretion from Xenopus myocytes and fibroblasts artificially loaded with acetylcholine is more prolonged and irregular compared to the release from neuronal growth cone (Girod et al., 1995).

Therefore, strategies have been adopted to differentiate spontaneous release in developing neurons from constitutive secretion in non neuronal cells. These mechanisms may include the storage of neurotransmitter in a class of vesicles, SVs, different from those involved in constitutive axonal membrane trafficking and the early expression of regulators of SV life cycle.

A developmental switch appears to modulate the competence of SVs to undergo recycling prior to synapse formation. Indeed, at early developmental stages SVs are reluctant to recycle both spontaneously and upon depolarization, whereas they begin to respond to depolarization at later stages. Importantly, the lack of responsiveness of SV to depolarizing stimuli at early stages of neuronal differentiation is not due to a limited Ca^{2+} influx (See Menegon et al., 2002), but rather to a lower sensitivity of the machinery involved in the translation of Ca^{2+} signals into the modulation of SV exocytosis. At synapses, a central component of this machinery is CaMKII (Soderling,

1999), which is thought to play a major role in the control of synapsin I function in the mature nerve terminals (Greengard et al., 1993). At variance, depolarization-dependent activation of CaMKII, and consequently synapsin I phopshorylation, are silenced in developing axons, due to a differential balance between kinase and phosphatase activities in this compartment. Refractoriness of CaMKII activation can be rescued using stimulating protocols that induce supraphysiological increases in intracellular Ca^{2+} levels, resulting in a concomitant ability of the kinase to phosphorylate synapsin I. After the establishment of synaptic contacts, the presynaptic pool of the kinase displays an increasing level of activity and acquires the parallel ability to phosphorylate synapsin I (Figure 4.3; Menegon et al., 2002). Remarkably, synapsin phosphorylation by PKA is already detected at early stages of development and is maintained throughout neuronal differentiation. Another relevant difference between synapsin phophorylation by PKA and CamKII is that the former shows a basal level of activation whereas the latter does not.

Therefore, PKA modulates synapsin function in developing neurons in the absence of phosphorylation by CaMKII, whereas activation of the axonal/presynaptic pool of CaMKII, coupled to synapsin I phosphorylation, increases with development in culture in parallel with synaptogenesis. These observations suggest that at the onset of synaptogenesis SVs acquire the ability to undergo depolarization-induced exocytosis by the addition of the CaMKII-mediated regulation of synapsin function to the preexisting modulation operated by the PKA/synapsin pathway on SV dynamics. Intriguingly, site 1 is the only phosphorylation site of synapsins found in invertebrates. Thus, one could speculate that in the growth cone the same basic mechanisms of neurotransmitter release operate which are present in the simplest neuronal networks, which have then evolved into more complex regulatory machineries to meet the requirements of efficient stimulus-secretion coupling needed in higher organisms.

An interesting observation reported in this study is that in the growth cone synapsins associate with a subpopulation of the total SV pool undergoing more sustained basal recycling. This indicates that synapsins associate with newly formed SVs (generated upon endocytosis and therefore labelled during 15 min-incubation in the presence of the anti-synaptotagmin antibody). A possible functional significance of this observation is that synapsins can identify a more mature state of these vesicles, maybe dictated by the presence of a molecular marker which could act as a receptor for synapsin binding to the vesicle or a distinctive lipid composition. Interestingly, synapsin co-localization with SVs is strongly enhanced in synaptic varicosities. Moreover, it increases in growth cones during development (5 DIV), supporting the idea of a molecular change in the protein/lipid compositin of SVs which parallels their functional maturation and coincides with an increased affinity for synapsin (Figure 3.21 and data not shown).

4.2.3 Do synapsins convert a general apparatus for growth cone motility and guidance into a machinery selective for the control of SV dynamics?

Localization of SVs in the C domain requires an intact F-actin meshwork in the P domain. Elevation of intracellular cAMP leads to SV dispersion in the P domain and enhances basal SV recycling. These results may provide a functional explanation for the characteristic spatial organization of SVs in the growth cone. F-actin at the C-P domain interface has been proposed to impede a "cytoplasmic flow" which drives axonal specification (Da Silva and Dotti, 2002). Cytochalasin D-induced alteration of the F-actin meshwork allows microtubule and cytoplasmic components to enter the P domain (Forscher and Smith, 1988; Contestabile et al., 2003).

In general, actin has been shown to act as a negative regulator of exocytosis. Cortical actin filaments create a barrier that requires transient removal to allow vesicles to

undergo fusion with the plasma membrane. Although not supported by all studies (See Eitzen, 2003 for references), this simplified actin-barrier model provides an explanation of how actin-disrupting reagents stimulate vesicle exocytosis in a variety of systems, from pancreatic beta cells to neurons (Orci et al., 1972; Morales et al., 2000). Interestingly, in these conditions exocytosis can be enhanced in the absence of classical stimuli, suggesting that actin remodeling may indeed be a sufficient trigger for exocytosis. Moreover, disruption of cortical actin upon PKC activation promotes exocytosis in chromaffin cells (Vitale et al., 1995).

We observed that even relatively short exposures to cytochalasin D induce mobilization of both SVs and the endosomal marker syntaxin 13 from the C to the P domain (data not shown). This indicates that F-actin provides a non-specific mechanism to control organelle distribution in the growth cone. However, one could speculate that a system to regulate SV distribution/recycling has evolved from this organization. By anchoring SVs to F-actin, synapsins convert the general organization of the growth cone cytoskeleton into a device to control SV functions depending on the physiological state of the growth cone. Signals stimulating synapsin phopshorylation will detach synapsins from SVs thus allowing SVs to undergo mobilization and recycling.

According to previous reports, cAMP enhances axonal anterograde transport, eventually inducing a Ca^{2+}-independent redistribution of organelles into the P domain of the growth cone (Forscher et al., 1987; Hollenbeck, 1993). This effect was proposed to depend on the PKA-stimulated entry of microtubules and associated organelles into the P domain, possibly facilitated by cAMP-dependent loosing of the F-actin meshwork at the C-P domain interface (Forscher et al., 1987). To increase intracellular cAMP, we applied protocols milder than those employed to produce a general enhancement of axonal anterograde transport (Forscher et al., 1987; Hollenbeck, 1993). Under these conditions, we observed selective changes in the distribution of SVs, which were

accompanied by an enhancement in the rate of SV recycling. In contrast, the localization of syntaxin 13-positive endosomes in the distal axon was unaffected. No major modifications in F-actin organization and growth cone morphology were visible, arguing against a direct effect of cytoskeletal dynamics in the cAMP-stimulated changes in SV distribution.

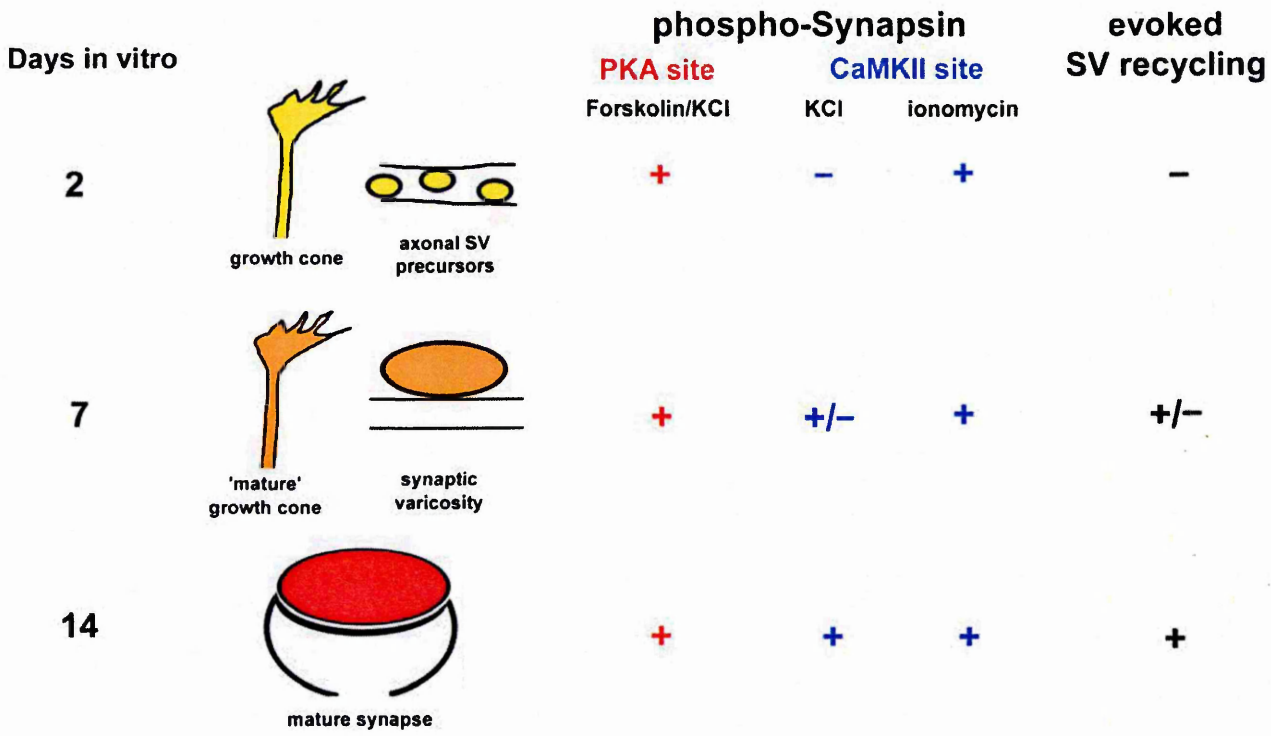

Figure 4.3. Does a phospho-switch regulate the responsiveness of SVs to depolarization-induced recycling during neuronal development?
Depolarization induces CaMKII-mediated phosphorylation of synapsin I at mature synapses (14 DIV) but not in the axon and growth cones at early developmental stages (2 DIV). At these early stages, SV recycling is insensitive to depolarization. CaMKII activation and the concomitant synapsin phosphorylation can be induced by supraphysiological increases in intracellular Ca^{2+} levels at all stages. At variance, PKA activation results in synapsin I phosphorylation at both early and late stages of neuronal development. The onset of CaMKII-mediated phosphorylation (DIV 7) coincides with the acquisition of evoked SV recycling at both isolated growth cones and synaptic varicosities.

It is conceivable that in the growth cone synapsins increase the selectivity and sensitivity of the response of SVs to cAMP variations. It is noteworthy that in growth cones cAMP mediates a variety of processes, including axon extension and navigation in response to diffusible factors (Song et al., 1997; Ming et al., 1997; Song et al., 1998; Sond and Poo, 1999). Synapsins may provide a mechanism to integrate the control of SV dynamics and functions with changes of cytoskeleton and trafficking underlying growth cone migration and guidance (Figure 4.2).

4.2.4 A role for the PKA/synapsin pathway in synapse formation

Synapsins appear to control the time-course of synapse formation during development. The precocious expression of synapsins leads to accelerated maturation of synapses formed in cultures (Lu et al., 1992; Schaeffer et al., 1994; Valtorta et al., 1995; Fiumara et al., 2004), whereas synapsin suppression delays neuronal differentiation, axonal extension and synaptogenesis (Chin et al., 1995; Ferreira et al., 1995). In this study I show that synapsin I and its phosphorylation by PKA play a pivotal role in regulating SV organization and dynamics in neuronal growth cones and in determining the formation of synaptic contacts. Overexpression of synapsin I strongly increases the number of synaptic contacts formed by hippocampal neurons in culture, and this effect is lost when the exogenously expressed synapsin I is mutated in the PKA-phosphorylation site. Since both wild-type and mutated synapsin I are able to retain SVs in the C domain, but only the wild-type molecule can disperse SVs throughout the growth cone and promote their recycling, these findings indicate that the formation and/or stabilization of synaptic contacts induced by synapsin I depends on its ability to respond to increases in intracellular cAMP, and not simply on a structural effect in the spatial organization of organelles in the growth cone.

How does the control of SV organization and recycling in the growth cones determine the capability of developing neurons to establish and/or maintain synaptic contacts?

SV exocytosis in the axons might be linked to the release of neurotransmitters (See Matteoli et al., 2004) which instruct axon pathfinding during the formation of neuronal networks. Alternatively, during the establishment of the synaptic contact, synapsins may direct the rapid reorganization of the SVs present in the C domain of the growth cone into the various functional pools of vesicles associated with the presynaptic terminal. Indeed, although a direct demonstration is lacking, SVs in the growth cone are likely to be used for the formation of the presynaptic pool of vesicles (Chow and Poo, 1985; Matteoli et al., 2004). Ahmari et al. (2000) showed that vesicles trasported along the isolated axon are recruited at the nascent synapse and rapidly form a recycling pool of SVs. These results indicate that SVs in the growth cone are required to build up a new functional synapse.

In addition, one could speculate that the usage of SVs in the isolated axon needs to be limited in order to maintain a pool of vesicles ready to generate a new synapse. Thus, similarly to what occurs in mature synapses, in developing neurons synapsins may provide a device to inhibit SV fusion at the resting state. Contact with the postsynaptic target retrogradely induces elevation in cAMP in the presynaptic compartment, and the consequent PKA-dependent increase in evoked Ca^{2+} influx (Funte and Haydon, 1993). It is tempting to speculate that during the formation of neuronal circuits, cAMP-dependent pathways control cone guidance, maybe regulating the release of transmitters from the growth cone, and, upon contact, elevation of cAMP triggers and chaperones the transformation of the growth cone into a mature neurosecretory terminals. Synapsins translate the cAMP increase into the control of SV distribution and recycling, thus allowing SVs to participate to the structural and functional changes underlying synapse formation.

It is also conceivable that the effects of synapsin on synapse formation rely on the ability of the protein to interact with and modulate the dynamics of the actin cytoskeleton (Valtorta et al., 1992). Indeed, changes in the organization of the actin network are known to play a relevant role during synaptogenesis (Goda and Davis, 2003).

The possibility that alterations in the synaptogenic activity of synapsin I lead to impairments in brain development and/or function is a testable hypothesis. Indeed, synapsin I-null mice exhibit spontaneous seizures (Li et al., 1995), and mutations in the synapsin I gene have been recently reported to cause epilepsy and/or mental retardation in humans (Garcia et al., 2004).

REFERENCES

Ahmari SE, Buchanan J, Smith SJ (2000) Assembly of presynaptic active zones from cytoplasmic transport packets. Nat Neurosci 3:445-451.

Alder J, Kanki H, Valtorta F, Greengard P, Poo MM (1995) Overexpression of synaptophysin enhances neurotransmitter secretion at xenopus neuromuscular synapses. J Neurosci 15:511-519.

Alder J, Lu B, Valtorta F, Greengard P, Poo MM (1992a) Calcium-dependent transmitter secretion reconstituted in xenopus oocytes: Requirement for synaptophysin. Science 257:657-661.

Alder J, Xie ZP, Valtorta F, Greengard P, Poo M (1992b) Antibodies to synaptophysin interfere with transmitter secretion at neuromuscular synapses. Neuron 9:759-768.

Ales E, Tabares L, Poyato JM, Valero V, Lindau M, Alvarez de Toledo G (1999) High calcium concentrations shift the mode of exocytosis to the kiss-and-run mechanism. Nat Cell Biol 1:40-44.

Almenar-Queralt A, Goldstein LS (2001) Linkers, packages and pathways: New concepts in axonal transport. Curr Opin Neurobiol 11:550-557.

Antonov I, Chang S, Zakharenko S, Popov SV (1999) Distribution of neurotransmitter secretion in growing axons. Neuroscience 90:975-984.

Asermely KE, Sterling GH, McCafferty MR, O'Neill JJ (1999) Synaptophysin is phosphorylated in rat cortical synaptosomes treated with botulinum toxin A. Life Sci 64:PL297-303.

Bacci A, Coco S, Pravettoni E, Schenk U, Armano S, Frassoni C, Verderio C, De Camilli P, Matteoli M (2001) Chronic blockade of glutamate receptors enhances presynaptic release and downregulates the interaction between synaptophysin-synaptobrevin-vesicle-associated membrane protein 2. J Neurosci 21:6588-6596.

Bahler M, Greengard P (1987) Synapsin I bundles F-actin in a phosphorylation-dependent manner. Nature 326:704-707.

Baitinger C, Willard M (1987) Axonal transport of synapsin I-like proteins in rabbit retinal ganglion cells. J Neurosci 7:3723-3735.

Bajjalieh SM, Frantz GD, Weimann JM, McConnell SK, Scheller RH (1994) Differential expression of synaptic vesicle protein 2 (SV2) isoforms. J Neurosci 14:5223-5235.

Barnekow A, Jahn R, Schartl M (1990) Synaptophysin: A substrate for the protein tyrosine kinase pp60c-src in intact synaptic vesicles. Oncogene 5:1019-1024.

Bastiaens PI, Squire A (1999) Fluorescence lifetime imaging microscopy: Spatial resolution of biochemical processes in the cell. Trends Cell Biol 9:48-52.

Bauerfeind R, Jelinek R, Hellwig A, Huttner WB (1995) Neurosecretory vesicles can be hybrids of synaptic vesicles and secretory granules. Proc Natl Acad Sci U S A 92:7342-7346.

Bauerfeind R, Regnier-Vigouroux A, Flatmark T, Huttner WB (1993) Selective storage of acetylcholine, but not catecholamines, in neuroendocrine synaptic-like microvesicles of early endosomal origin. Neuron 11:105-121.

Baumert M, Takei K, Hartinger J, Burger PM, Fischer von Mollard G, Maycox PR, De Camilli P, Jahn R (1990) P29: A novel tyrosine-phosphorylated membrane protein present in small clear vesicles of neurons and endocrine cells. J Cell Biol 110:1285-1294.

Becher A, Drenckhahn A, Pahner I, Margittai M, Jahn R, Ahnert-Hilger G (1999a) The synaptophysin-synaptobrevin complex: A hallmark of synaptic vesicle maturation. J Neurosci 19:1922-1931.

Becher A, Drenckhahn A, Pahner I, Ahnert-Hilger G (1999b) The synaptophysin-synaptobrevin complex is developmentally upregulated in cultivated neurons but is absent in neuroendocrine cells. Eur J Cell Biol 78:650-656.

Belfort GM, Bakirtzi K, Kandror KV (2005) Cellugyrin induces biogenesis of synaptic-like microvesicles in PC12 cells. J Biol Chem 280:7262-7272.

Belfort GM, Kandror KV (2003) Cellugyrin and synaptogyrin facilitate targeting of synaptophysin to a ubiquitous synaptic vesicle-sized compartment in PC12 cells. J Biol Chem 278:47971-47978.

Benfenati F, Valtorta F, Chieregatti E, Greengard P (1992) Interaction of free and synaptic vesicle-bound synapsin I with F-actin. Neuron 8:377-386.

Benfenati F, Neyroz P, Bahler M, Masotti L, Greengard P (1990) Time-resolved fluorescence study of the neuron-specific phosphoprotein synapsin I. evidence for phosphorylation-dependent conformational changes. J Biol Chem 265:12584-12595.

Benfenati F, Bahler M, Jahn R, Greengard P (1989) Interactions of synapsin I with small synaptic vesicles: Distinct sites in synapsin I bind to vesicle phospholipids and vesicle proteins. J Cell Biol 108:1863-1872.

Bennett MK, Calakos N, Kreiner T, Scheller RH (1992) Synaptic vesicle membrane proteins interact to form a multimeric complex. J Cell Biol 116:761-775.

Betz WJ, Mao F, Smith CB (1996) Imaging exocytosis and endocytosis. Curr Opin Neurobiol 6:365-371.

Betz WJ, Henkel AW (1994) Okadaic acid disrupts clusters of synaptic vesicles in frog motor nerve terminals. J Cell Biol 124:843-854.

Betz H (1990) Homology and analogy in transmembrane channel design: Lessons from synaptic membrane proteins. Biochemistry 29:3591-3599.

Blagoveshchenskaya AD, Cutler DF (2000) Sorting to synaptic-like microvesicles from early and late endosomes requires overlapping but not identical targeting signals. Mol Biol Cell 11:1801-1814.

Blagoveshchenskaya AD, Hewitt EW, Cutler DF (1999a) Di-leucine signals mediate targeting of tyrosinase and synaptotagmin to synaptic-like microvesicles within PC12 cells. Mol Biol Cell 10:3979-3990.

Blagoveshchenskaya AD, Hewitt EW, Cutler DF (1999b) A complex web of signal-dependent trafficking underlies the triorganellar distribution of P-selectin in neuroendocrine PC12 cells. J Cell Biol 145:1419-1433.

Blondeau F, Ritter B, Allaire PD, Wasiak S, Girard M, Hussain NK, Angers A, Legendre-Guillemin V, Roy L, Boismenu D, Kearney RE, Bell AW, Bergeron JJ, McPherson PS (2004) Tandem MS analysis of brain clathrin-coated vesicles reveals their critical involvement in synaptic vesicle recycling. Proc Natl Acad Sci U S A 101:3833-3838.

Blumstein J, Faundez V, Nakatsu F, Saito T, Ohno H, Kelly RB (2001) The neuronal form of adaptor protein-3 is required for synaptic vesicle formation from endosomes. J Neurosci 21:8034-8042.

Bonanomi D, Pennuto M, Rigoni M, Rossetto O, Montecucco C, Valtorta F (2005) Taipoxin induces synaptic vesicle exocytosis and disrupts the interaction of synaptophysin I with VAMP2. Mol Pharmacol 67:1901-1908.

Bonifacino JS, Traub LM (2003) Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu Rev Biochem 72:395-447.

Bonzelius F, Herman GA, Cardone MH, Mostov KE, Kelly RB (1994) The polymeric immunoglobulin receptor accumulates in specialized endosomes but not synaptic vesicles within the neurites of transfected neuroendocrine PC12 cells. J Cell Biol 127:1603-1616.

Booher J, Sensenbrenner M (1972) Growth and cultivation of dissociated neurons and glial cells from embryonic chick, rat and human brain in flask cultures. Neurobiology 2:97-105.

Brooks CC, Scherer PE, Cleveland K, Whittemore JL, Lodish HF, Cheatham B (2000) Pantophysin is a phosphoprotein component of adipocyte transport vesicles and associates with GLUT4-containing vesicles. J Biol Chem 275:2029-2036.

Brose N, Petrenko AG, Sudhof TC, Jahn R (1992) Synaptotagmin: A calcium sensor on the synaptic vesicle surface. Science 256:1021-1025.

Browning MD, Dudek EM (1992) Activators of protein kinase C increase the phosphorylation of the synapsins at sites phosphorylated by cAMP-dependent and Ca2+/calmodulin-dependent protein kinase in the rat hippocampal slice. Synapse 10:62-70.

Bucci C, Parton RG, Mather IH, Stunnenberg H, Simons K, Hoflack B, Zerial M (1992) The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. Cell 70:715-728.

Buckley KM, Floor E, Kelly RB (1987) Cloning and sequence analysis of cDNA encoding p38, a major synaptic vesicle protein. J Cell Biol 105:2447-2456.

Calakos N, Scheller RH (1994) Vesicle-associated membrane protein and synaptophysin are associated on the synaptic vesicle. J Biol Chem 269:24534-24537.

Cameron PL, Sudhof TC, Jahn R, De Camilli P (1991) Colocalization of synaptophysin with transferrin receptors: Implications for synaptic vesicle biogenesis. J Cell Biol 115:151-164.

Carrion-Vazquez M, Fernandez AM, Chowen J, Nieto-Sampedro M (1998) Brain Ac39/physophilin: Cloning, coexpression and colocalization with synaptophysin. Eur J Neurosci 10:1153-1166.

Ceccaldi PE, Grohovaz F, Benfenati F, Chieregatti E, Greengard P, Valtorta F (1995) Dephosphorylated synapsin I anchors synaptic vesicles to actin cytoskeleton: An analysis by videomicroscopy. J Cell Biol 128:905-912.

Ceccarelli B, Hurlbut WP (1980) Ca2+-dependent recycling of synaptic vesicles at the frog neuromuscular junction. J Cell Biol 87:297-303.

Ceccarelli B, Hurlbut WP, Mauro A (1972) Depletion of vesicles from frog neuromuscular junctions by prolonged tetanic stimulation. J Cell Biol 54:30-38.

Ceccarelli B, Hurlbut WP, Mauro A (1973) Turnover of transmitter and synaptic vesicles at the frog neuromuscular junction. J Cell Biol 57:499-524.

Chamberlain LH, Burgoyne RD, Gould GW (2001) SNARE proteins are highly enriched in lipid rafts in PC12 cells: Implications for the spatial control of exocytosis. Proc Natl Acad Sci U S A 98:5619-5624.

Chang S, De Camilli P (2001) Glutamate regulates actin-based motility in axonal filopodia. Nat Neurosci 4:787-793.

Chi P, Greengard P, Ryan TA (2003) Synaptic vesicle mobilization is regulated by distinct synapsin I phosphorylation pathways at different frequencies. Neuron 38:69-78.

Chi P, Greengard P, Ryan TA (2001) Synapsin dispersion and reclustering during synaptic activity. Nat Neurosci 4:1187-1193.

Chin LS, Li L, Ferreira A, Kosik KS, Greengard P (1995) Impairment of axonal development and of synaptogenesis in hippocampal neurons of synapsin I-deficient mice. Proc Natl Acad Sci U S A 92:9230-9234.

Chow I, Poo MM (1985) Release of acetylcholine from embryonic neurons upon contact with muscle cell. J Neurosci 5:1076-1082.

Christoforidis S, McBride HM, Burgoyne RD, Zerial M (1999) The Rab5 effector EEA1 is a core component of endosome docking. Nature 397:621-625.

Clift-O'Grady L, Linstedt AD, Lowe AW, Grote E, Kelly RB (1990) Biogenesis of synaptic vesicle-like structures in a pheochromocytoma cell line PC-12. J Cell Biol 110:1693-1703.

Conner SD, Schmid SL (2003) Regulated portals of entry into the cell. Nature 422:37-44.

Contestabile A, Bonanomi D, Burgaya F, Girault JA, Valtorta F (2003) Localization of focal adhesion kinase isoforms in cells of the central nervous system. Int J Dev Neurosci 21:83-93.

Cousin MA, Robinson PJ (2000) Two mechanisms of synaptic vesicle recycling in rat brain nerve terminals. J Neurochem 75:1645-1653.

Cremona O, De Camilli P (1997) Synaptic vesicle endocytosis. Curr Opin Neurobiol 7:323-330.

da Silva JS, Dotti CG (2002) Breaking the neuronal sphere: Regulation of the actin cytoskeleton in neuritogenesis. Nat Rev Neurosci 3:694-704.

Daly C, Ziff EB (2002) Ca2+-dependent formation of a dynamin-synaptophysin complex: Potential role in synaptic vesicle endocytosis. J Biol Chem 277:9010-9015.

Daly C, Sugimori M, Moreira JE, Ziff EB, Llinas R (2000) Synaptophysin regulates clathrin-independent endocytosis of synaptic vesicles. Proc Natl Acad Sci U S A 97:6120-6125.

Damke H, Baba T, Warnock DE, Schmid SL (1994) Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. J Cell Biol 127:915-934.

Dan Y, Poo MM (1992) Quantal transmitter secretion from myocytes loaded with acetylcholine. Nature 359:733-736.

De Camilli P, Takei K (1996) Molecular mechanisms in synaptic vesicle endocytosis and recycling. Neuron 16:481-486.

De Camilli P, Benfenati F, Valtorta F, Greengard P (1990) The synapsins. Annu Rev Cell Biol 6:433-460.

De Camilli P, Cameron R, Greengard P (1983) Synapsin I (protein I), a nerve terminal-specific phosphoprotein. I. its general distribution in synapses of the central and peripheral nervous system demonstrated by immunofluorescence in frozen and plastic sections. J Cell Biol 96:1337-1354.

de Hoop MJ, Huber LA, Stenmark H, Williamson E, Zerial M, Parton RG, Dotti CG (1994) The involvement of the small GTP-binding protein Rab5a in neuronal endocytosis. Neuron 13:11-22.

de Lange RP, de Roos AD, Borst JG (2003) Two modes of vesicle recycling in the rat calyx of held. J Neurosci 23:10164-10173.

De Paola V, Arber S, Caroni P (2003) AMPA receptors regulate dynamic equilibrium of presynaptic terminals in mature hippocampal networks. Nat Neurosci 6:491-500.

de Wit H, Lichtenstein Y, Kelly RB, Geuze HJ, Klumperman J, van der Sluijs P (2001) Rab4 regulates formation of synaptic-like microvesicles from early endosomes in PC12 cells. Mol Biol Cell 12:3703-3715.

de Wit H, Lichtenstein Y, Geuze HJ, Kelly RB, van der Sluijs P, Klumperman J (1999) Synaptic vesicles form by budding from tubular extensions of sorting endosomes in PC12 cells. Mol Biol Cell 10:4163-4176.

Deak F, Schoch S, Liu X, Sudhof TC, Kavalali ET (2004) Synaptobrevin is essential for fast synaptic-vesicle endocytosis. Nat Cell Biol 6:1102-1108.

Demarque M, Represa A, Becq H, Khalilov I, Ben-Ari Y, Aniksztejn L (2002) Paracrine intercellular communication by a Ca2+- and SNARE-independent release of GABA and glutamate prior to synapse formation. Neuron 36:1051-1061.

Desnos C, Clift-O'Grady L, Kelly RB (1995) Biogenesis of synaptic vesicles in vitro. J Cell Biol 130:1041-1049.

Diefenbach TJ, Guthrie PB, Stier H, Billups B, Kater SB (1999) Membrane recycling in the neuronal growth cone revealed by FM1-43 labeling. J Neurosci 19:9436-9444.

Dunlap DD, Valtorta F (2003) An Image Pro-Plus macro for FRET measurement. Media Cybernetics Application Note.

Eaton BA, Haugwitz M, Lau D, Moore HP (2000) Biogenesis of regulated exocytotic carriers in neuroendocrine cells. J Neurosci 20:7334-7344.

Edelmann L, Hanson PI, Chapman ER, Jahn R (1995) Synaptobrevin binding to synaptophysin: A potential mechanism for controlling the exocytotic fusion machine. EMBO J 14:224-231.

Eitzen G (2003) Actin remodeling to facilitate membrane fusion. Biochim Biophys Acta 1641:175-181.

Eshkind LG, Leube RE (1995) Mice lacking synaptophysin reproduce and form typical synaptic vesicles. Cell Tissue Res 282:423-433.

Esser L, Wang CR, Hosaka M, Smagula CS, Sudhof TC, Deisenhofer J (1998) Synapsin I is structurally similar to ATP-utilizing enzymes. EMBO J 17:977-984.

Evers J, Laser M, Sun YA, Xie ZP, Poo MM (1989) Studies of nerve-muscle interactions in xenopus cell culture: Analysis of early synaptic currents. J Neurosci 9:1523-1539.

Faundez V, Horng JT, Kelly RB (1998) A function for the AP3 coat complex in synaptic vesicle formation from endosomes. Cell 93:423-432.

Faundez V, Horng JT, Kelly RB (1997) ADP ribosylation factor 1 is required for synaptic vesicle budding in PC12 cells. J Cell Biol 138:505-515.

Feany MB, Yee AG, Delvy ML, Buckley KM (1993) The synaptic vesicle proteins SV2, synaptotagmin and synaptophysin are sorted to separate cellular compartments in CHO fibroblasts. J Cell Biol 123:575-584.

Feng J, Chi P, Blanpied TA, Xu Y, Magarinos AM, Ferreira A, Takahashi RH, Kao HT, McEwen BS, Ryan TA, Augustine GJ, Greengard P (2002) Regulation of neurotransmitter release by synapsin III. J Neurosci 22:4372-4380.

Fergestad T, Broadie K (2001) Interaction of stoned and synaptotagmin in synaptic vesicle endocytosis. J Neurosci 21:1218-1227.

Fernandez-Chacon R, Sudhof TC (2000) Novel SCAMPs lacking NPF repeats: Ubiquitous and synaptic vesicle-specific forms implicate SCAMPs in multiple membrane-trafficking functions. J Neurosci 20:7941-7950.

Ferreira A, Kao HT, Feng J, Rapoport M, Greengard P (2000) Synapsin III: Developmental expression, subcellular localization, and role in axon formation. J Neurosci 20:3736-3744.

Ferreira A, Chin LS, Li L, Lanier LM, Kosik KS, Greengard P (1998) Distinct roles of synapsin I and synapsin II during neuronal development. Mol Med 4:22-28.

Ferreira A, Han HQ, Greengard P, Kosik KS (1995) Suppression of synapsin II inhibits the formation and maintenance of synapses in hippocampal culture. Proc Natl Acad Sci U S A 92:9225-9229.

Fesce R, Meldolesi J (1999) Peeping at the vesicle kiss. Nat Cell Biol 1:E3-4.

Fesce R, Grohovaz F, Valtorta F, Meldolesi J (1994) Neurotransmitter release: Fusion or 'kiss-and-run'? Trends Cell Biol 4:1-4.

Fischer von Mollard G, Stahl B, Walch-Solimena C, Takei K, Daniels L, Khoklatchev A, De Camilli P, Sudhof TC, Jahn R (1994) Localization of Rab5 to synaptic vesicles identifies endosomal intermediate in synaptic vesicle recycling pathway. Eur J Cell Biol 65:319-326.

Fiumara F, Onofri F, Benfenati F, Montarolo PG, Ghirardi M (2001) Intracellular injection of synapsin I induces neurotransmitter release in C1 neurons of helix pomatia contacting a wrong target. Neuroscience 104:271-280.

Fletcher TL, Cameron P, De Camilli P, Banker G (1991) The distribution of synapsin I and synaptophysin in hippocampal neurons developing in culture. J Neurosci 11:1617-1626.

Fohlman J, Eaker D, Karlsoon E, Thesleff S (1976) Taipoxin, an extremely potent presynaptic neurotoxin from the venom of the australian snake taipan (oxyuranus s. scutellatus). isolation, characterization, quaternary structure and pharmacological properties. Eur J Biochem 68:457-469.

Forscher P, Smith SJ (1988) Actions of cytochalasins on the organization of actin filaments and microtubules in a neuronal growth cone. J Cell Biol 107:1505-1516.

Forscher P, Kaczmarek LK, Buchanan JA, Smith SJ (1987) Cyclic AMP induces changes in distribution and transport of organelles within growth cones of aplysia bag cell neurons. J Neurosci 7:3600-3611.

Friedman HV, Bresler T, Garner CC, Ziv NE (2000) Assembly of new individual excitatory synapses: Time course and temporal order of synaptic molecule recruitment. Neuron 27:57-69.

Funte LR, Haydon PG (1993) Synaptic target contact enhances presynaptic calcium influx by activating cAMP-dependent protein kinase during synaptogenesis. Neuron 10:1069-1078.

Fykse EM, Takei K, Walch-Solimena C, Geppert M, Jahn R, De Camilli P, Sudhof TC (1993) Relative properties and localizations of synaptic vesicle protein isoforms: The case of the synaptophysins. J Neurosci 13:4997-5007.

Gad H, Low P, Zotova E, Brodin L, Shupliakov O (1998) Dissociation between Ca2+-triggered synaptic vesicle exocytosis and clathrin-mediated endocytosis at a central synapse. Neuron 21:607-616.

Galli T, McPherson PS, De Camilli P (1996) The V0 sector of the V-ATPase, synaptobrevin, and synaptophysin are associated on synaptic vesicles in a triton X-100-resistant, freeze-thawing sensitive, complex. J Biol Chem 271:2193-2198.

Gao XB, van den Pol AN (2000) GABA release from mouse axonal growth cones. J Physiol 523 Pt 3:629-637.

Garcia CC, Blair HJ, Seager M, Coulthard A, Tennant S, Buddles M, Curtis A, Goodship JA (2004) Identification of a mutation in synapsin I, a synaptic vesicle protein, in a family with epilepsy. J Med Genet 41:183-186.

Gaspary HL, Wang W, Richerson GB (1998) Carrier-mediated GABA release activates GABA receptors on hippocampal neurons. J Neurophysiol 80:270-281.

Geppert M, Goda Y, Stevens CF, Sudhof TC (1997) The small GTP-binding protein Rab3A regulates a late step in synaptic vesicle fusion. Nature 387:810-814.

Geppert M, Goda Y, Hammer RE, Li C, Rosahl TW, Stevens CF, Sudhof TC (1994) Synaptotagmin I: A major Ca2+ sensor for transmitter release at a central synapse. Cell 79:717-727.

Gil C, Soler-Jover A, Blasi J, Aguilera J (2005) Synaptic proteins and SNARE complexes are localized in lipid rafts from rat brain synaptosomes. Biochem Biophys Res Commun 329:117-124.

Giovedi S, Darchen F, Valtorta F, Greengard P, Benfenati F (2004a) Synapsin is a novel Rab3 effector protein on small synaptic vesicles. II. functional effects of the Rab3A-synapsin I interaction. J Biol Chem 279:43769-43779.

Giovedi S, Vaccaro P, Valtorta F, Darchen F, Greengard P, Cesareni G, Benfenati F (2004b) Synapsin is a novel Rab3 effector protein on small synaptic vesicles. I. identification and characterization of the synapsin I-Rab3 interactions in vitro and in intact nerve terminals. J Biol Chem 279:43760-43768.

Girod R, Popov S, Alder J, Zheng JQ, Lohof A, Poo MM (1995) Spontaneous quantal transmitter secretion from myocytes and fibroblasts: Comparison with neuronal secretion. J Neurosci 15:2826-2838.

Gitler D, Xu Y, Kao HT, Lin D, Lim S, Feng J, Greengard P, Augustine GJ (2004) Molecular determinants of synapsin targeting to presynaptic terminals. J Neurosci 24:3711-3720.

Goda Y, Davis GW (2003) Mechanisms of synapse assembly and disassembly. Neuron 40:243-264.

Goldstein LS (2003) Do disorders of movement cause movement disorders and dementia? Neuron 40:415-425.

Gonzalez-Gaitan M, Jackle H (1997) Role of drosophila alpha-adaptin in presynaptic vesicle recycling. Cell 88:767-776.

Graham ME, Burgoyne RD (2000) Comparison of cysteine string protein (csp) and mutant alpha-SNAP overexpression reveals a role for csp in late steps of membrane fusion in dense-core granule exocytosis in adrenal chromaffin cells. J Neurosci 20:1281-1289.

Grass I, Thiel S, Honing S, Haucke V (2004) Recognition of a basic AP-2 binding motif within the C2B domain of synaptotagmin is dependent on multimerization. J Biol Chem 279:54872-54880.

Greengard P, Valtorta F, Czernik AJ, Benfenati F (1993) Synaptic vesicle phosphoproteins and regulation of synaptic function. Science 259:780-785.

Grote E, Kelly RB (1996) Endocytosis of VAMP is facilitated by a synaptic vesicle targeting signal. J Cell Biol 132:537-547.

Grote E, Hao JC, Bennett MK, Kelly RB (1995) A targeting signal in VAMP regulating transport to synaptic vesicles. Cell 81:581-589.

Gundelfinger ED, Kessels MM, Qualmann B (2003) Temporal and spatial coordination of exocytosis and endocytosis. Nat Rev Mol Cell Biol 4:127-139.

Haass NK, Kartenbeck MA, Leube RE (1996) Pantophysin is a ubiquitously expressed synaptophysin homologue and defines constitutive transport vesicles. J Cell Biol 134:731-746.

Hackett JT, Cochran SL, Greenfield LJ,Jr, Brosius DC, Ueda T (1990) Synapsin I injected presynaptically into goldfish mauthner axons reduces quantal synaptic transmission. J Neurophysiol 63:701-706.

Han HQ, Greengard P (1994) Remodeling of cytoskeletal architecture of nonneuronal cells induced by synapsin. Proc Natl Acad Sci U S A 91:8557-8561.

Han HQ, Nichols RA, Rubin MR, Bahler M, Greengard P (1991) Induction of formation of presynaptic terminals in neuroblastoma cells by synapsin IIb. Nature 349:697-700.

Han W, Rhee JS, Maximov A, Lao Y, Mashimo T, Rosenmund C, Sudhof TC (2004) N-glycosylation is essential for vesicular targeting of synaptotagmin 1. Neuron 41:85-99.

Hannah MJ, Schmidt AA, Huttner WB (1999) Synaptic vesicle biogenesis. Annu Rev Cell Dev Biol 15:733-798.

Haucke V, Wenk MR, Chapman ER, Farsad K, De Camilli P (2000) Dual interaction of synaptotagmin with mu2- and alpha-adaptin facilitates clathrin-coated pit nucleation. EMBO J 19:6011-6019.

Haucke V, De Camilli P (1999) AP-2 recruitment to synaptotagmin stimulated by tyrosine-based endocytic motifs. Science 285:1268-1271.

Heuser JE, Reese TS (1973) Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. J Cell Biol 57:315-344.

Hilfiker S, Benfenati F, Doussau F, Nairn AC, Czernik AJ, Augustine GJ, Greengard P (2005) Structural domains involved in the regulation of transmitter release by synapsins. J Neurosci 25:2658-2669.

Hilfiker S, Pieribone VA, Czernik AJ, Kao HT, Augustine GJ, Greengard P (1999) Synapsins as regulators of neurotransmitter release. Philos Trans R Soc Lond B Biol Sci 354:269-279.

Hilfiker S, Schweizer FE, Kao HT, Czernik AJ, Greengard P, Augustine GJ (1998) Two sites of action for synapsin domain E in regulating neurotransmitter release. Nat Neurosci 1:29-35.

Hirling H, Scheller RH (1996) Phosphorylation of synaptic vesicle proteins: Modulation of the alpha SNAP interaction with the core complex. Proc Natl Acad Sci U S A 93:11945-11949.

Hollenbeck PJ (1993) Products of endocytosis and autophagy are retrieved from axons by regulated retrograde organelle transport. J Cell Biol 121:305-315.

Holt M, Cooke A, Wu MM, Lagnado L (2003) Bulk membrane retrieval in the synaptic terminal of retinal bipolar cells. J Neurosci 23:1329-1339.

Holtta-Vuori M, Tanhuanpaa K, Mobius W, Somerharju P, Ikonen E (2002) Modulation of cellular cholesterol transport and homeostasis by Rab11. Mol Biol Cell 13:3107-3122.

Horikawa HP, Kneussel M, El Far O, Betz H (2002) Interaction of synaptophysin with the AP-1 adaptor protein gamma-adaptin. Mol Cell Neurosci 21:454-462.

Hosaka M, Sudhof TC (1999) Homo- and heterodimerization of synapsins. J Biol Chem 274:16747-16753.

Hosaka M, Hammer RE, Sudhof TC (1999) A phospho-switch controls the dynamic association of synapsins with synaptic vesicles. Neuron 24:377-387.

Hosaka M, Sudhof TC (1998) Synapsins I and II are ATP-binding proteins with differential Ca2+ regulation. J Biol Chem 273:1425-1429.

Hubner K, Windoffer R, Hutter H, Leube RE (2002) Tetraspan vesicle membrane proteins: Synthesis, subcellular localization, and functional properties. Int Rev Cytol 214:103-159.

Hume RI, Role LW, Fischbach GD (1983) Acetylcholine release from growth cones detected with patches of acetylcholine receptor-rich membranes. Nature 305:632-634.

Humeau Y, Doussau F, Vitiello F, Greengard P, Benfenati F, Poulain B (2001) Synapsin controls both reserve and releasable synaptic vesicle pools during neuronal activity and short-term plasticity in aplysia. J Neurosci 21:4195-4206.

Huttner WB, Schmidt A (2000) Lipids, lipid modification and lipid-protein interaction in membrane budding and fission--insights from the roles of endophilin A1 and synaptophysin in synaptic vesicle endocytosis. Curr Opin Neurobiol 10:543-551.

Huttner WB, Schiebler W, Greengard P, De Camilli P (1983) Synapsin I (protein I), a nerve terminal-specific phosphoprotein. III. its association with synaptic vesicles studied in a highly purified synaptic vesicle preparation. J Cell Biol 96:1374-1388.

Jacob R, Naim HY (2001) Apical membrane proteins are transported in distinct vesicular carriers. Curr Biol 11:1444-1450.

Jahn R, Schiebler W, Ouimet C, Greengard P (1985) A 38,000-dalton membrane protein (p38) present in synaptic vesicles. Proc Natl Acad Sci U S A 82:4137-4141.

Janz R, Sudhof TC, Hammer RE, Unni V, Siegelbaum SA, Bolshakov VY (1999) Essential roles in synaptic plasticity for synaptogyrin I and synaptophysin I. Neuron 24:687-700.

Janz R, Sudhof TC (1998) Cellugyrin, a novel ubiquitous form of synaptogyrin that is phosphorylated by pp60c-src. J Biol Chem 273:2851-2857.

Jarousse N, Kelly RB (2001) The AP2 binding site of synaptotagmin 1 is not an internalization signal but a regulator of endocytosis. J Cell Biol 154:857-866.

Johnson EM, Ueda T, Maeno H, Greengard P (1997) Adenosine 3',5-monophosphate-dependent phosphorylation of a specific protein in synaptic membrane fractions from rat cerebrum. J Biol Chem 247:5650-5652.

Johnston PA, Sudhof TC (1990) The multisubunit structure of synaptophysin. relationship between disulfide bonding and homo-oligomerization. J Biol Chem 265:8869-8873.

Johnston PA, Cameron PL, Stukenbrok H, Jahn R, De Camilli P, Sudhof TC (1989) Synaptophysin is targeted to similar microvesicles in CHO and PC12 cells. EMBO J 8:2863-2872.

Jorgensen EM, Hartwieg E, Schuske K, Nonet ML, Jin Y, Horvitz HR (1995) Defective recycling of synaptic vesicles in synaptotagmin mutants of caenorhabditis elegans. Nature 378:196-199.

Jovanovic JN, Sihra TS, Nairn AC, Hemmings HC,Jr, Greengard P, Czernik AJ (2001) Opposing changes in phosphorylation of specific sites in synapsin I during Ca2+-dependent glutamate release in isolated nerve terminals. J Neurosci 21:7944-7953.

Jovanovic JN, Benfenati F, Siow YL, Sihra TS, Sanghera JS, Pelech SL, Greengard P, Czernik AJ (1996) Neurotrophins stimulate phosphorylation of synapsin I by MAP kinase and regulate synapsin I-actin interactions. Proc Natl Acad Sci U S A 93:3679-3683.

Jovin TM, Arndt-Jovin DJ (1989) Luminescence digital imaging microscopy. Annu Rev Biophys Biophys Chem 18:271-308.

Kaether C, Skehel P, Dotti CG (2000) Axonal membrane proteins are transported in distinct carriers: A two-color video microscopy study in cultured hippocampal neurons. Mol Biol Cell 11:1213-1224.

Kamal A, Stokin GB, Yang Z, Xia CH, Goldstein LS (2000) Axonal transport of amyloid precursor protein is mediated by direct binding to the kinesin light chain subunit of kinesin-I. Neuron 28:449-459.

Kamenskaya MA, Thesleff S (1974) The neuromuscular blocking action of an isolated toxin from the elapid (oxyuranus scutellactus). Acta Physiol Scand 90:716-724.

Kanaani J, Diacovo MJ, El-Husseini A, Bredt DS, Baekkeskov S (2004) Palmitoylation controls trafficking of GAD65 from golgi membranes to axon-specific endosomes and a Rab5a-dependent pathway to presynaptic clusters. J Cell Sci 117:2001-2013.

Kanaani J, el-Husseini A, Aguilera-Moreno A, Diacovo JM, Bredt DS, Baekkeskov S (2002) A combination of three distinct trafficking signals mediates axonal targeting and presynaptic clustering of GAD65. J Cell Biol 158:1229-1238.

Kaneko M, Takahashi T (2004) Presynaptic mechanism underlying cAMP-dependent synaptic potentiation. J Neurosci 24:5202-5208.

Kang R, Swayze R, Lise MF, Gerrow K, Mullard A, Honer WG, El-Husseini A (2004) Presynaptic trafficking of synaptotagmin I is regulated by protein palmitoylation. J Biol Chem 279:50524-50536.

Kantheti P, Qiao X, Diaz ME, Peden AA, Meyer GE, Carskadon SL, Kapfhamer D, Sufalko D, Robinson MS, Noebels JL, Burmeister M (1998) Mutation in AP-3 delta in the mocha mouse links endosomal transport to storage deficiency in platelets, melanosomes, and synaptic vesicles. Neuron 21:111-122.

Kao HT, Song HJ, Porton B, Ming GL, Hoh J, Abraham M, Czernik AJ, Pieribone VA, Poo MM, Greengard P (2002) A protein kinase A-dependent molecular switch in synapsins regulates neurite outgrowth. Nat Neurosci 5:431-437.

Kao HT, Porton B, Hilfiker S, Stefani G, Pieribone VA, DeSalle R, Greengard P (1999) Molecular evolution of the synapsin gene family. J Exp Zool 285:360-377.

Keller P, Simons K (1998) Cholesterol is required for surface transport of influenza virus hemagglutinin. J Cell Biol 140:1357-1367.

Keller P, Toomre D, Diaz E, White J, Simons K (2001) Multicolour imaging of post-golgi sorting and trafficking in live cells. Nat Cell Biol 3:140-149.

Kelly RB (1993) Secretion. A question of endosomes. Nature 364:487-488.

Kim PK, Hollerbach C, Trimble WS, Leber B, Andrews DW (1999) Identification of the endoplasmic reticulum targeting signal in vesicle-associated membrane proteins. J Biol Chem 274:36876-36882.

Kini RM (2003) Excitement ahead: Structure, function and mechanism of snake venom phospholipase A2 enzymes. Toxicon 42:827-840.

Klingauf J, Kavalali ET, Tsien RW (1998) Kinetics and regulation of fast endocytosis at hippocampal synapses. Nature 394:581-585.

Klumperman J, Hille A, Veenendaal T, Oorschot V, Stoorvogel W, von Figura K, Geuze HJ (1993) Differences in the endosomal distributions of the two mannose 6-phosphate receptors. J Cell Biol 121:997-1010.

Knaus P, Marqueze-Pouey B, Scherer H, Betz H (1990) Synaptoporin, a novel putative channel protein of synaptic vesicles. Neuron 5:453-462.

Koenig JH, Ikeda K (1996) Synaptic vesicles have two distinct recycling pathways. J Cell Biol 135:797-808.

Koenig JH, Ikeda K (1989) Disappearance and reformation of synaptic vesicle membrane upon transmitter release observed under reversible blockage of membrane retrieval. J Neurosci 9:3844-3860.

Koh TW, Bellen HJ (2003) Synaptotagmin I, a Ca2+ sensor for neurotransmitter release. Trends Neurosci 26:413-422.

Kosik KS, Finch EA (1987) MAP2 and tau segregate into dendritic and axonal domains after the elaboration of morphologically distinct neurites: An immunocytochemical study of cultured rat cerebrum. J Neurosci 7:3142-3153.

Kraszewski K, Mundigl O, Daniell L, Verderio C, Matteoli M, De Camilli P (1995) Synaptic vesicle dynamics in living cultured hippocampal neurons visualized with CY3-conjugated antibodies directed against the lumenal domain of synaptotagmin. J Neurosci 15:4328-4342.

Kuromi H, Kidokoro Y (1998) Two distinct pools of synaptic vesicles in single presynaptic boutons in a temperature-sensitive drosophila mutant, shibire. Neuron 20:917-925.

Kutay U, Ahnert-Hilger G, Hartmann E, Wiedenmann B, Rapoport TA (1995) Transport route for synaptobrevin via a novel pathway of insertion into the endoplasmic reticulum membrane. EMBO J 14:217-223.

Kutay U, Hartmann E, Rapoport TA (1993) A class of membrane proteins with a C-terminal anchor. Trends Cell Biol 3:72-75.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lah JJ, Burry RW (1993) Synaptophysin has a selective distribution in early endosomes of PC12 cells. J Neurocytol 22:92-101.

Lang T, Bruns D, Wenzel D, Riedel D, Holroyd P, Thiele C, Jahn R (2001) SNAREs are concentrated in cholesterol-dependent clusters that define docking and fusion sites for exocytosis. EMBO J 20:2202-2213.

Ledesma MD, Brugger B, Bunning C, Wieland FT, Dotti CG (1999) Maturation of the axonal plasma membrane requires upregulation of sphingomyelin synthesis and formation of protein-lipid complexes. EMBO J 18:1761-1771.

Ledesma MD, Simons K, Dotti CG (1998) Neuronal polarity: Essential role of protein-lipid complexes in axonal sorting. Proc Natl Acad Sci U S A 95:3966-3971.

Leenders AGM, Scholten G, De Lange RPJ, Lopes da Silva FH, Ghijsen WEJM (2002) Sequential changes in synaptic vesicle pools and endsome-like organelles during depolarization near the active zone of central nerve terminals. Neuroscience 109:195-206.

Leoni C, Menegon A, Benfenati F, Toniolo D, Pennuto M, Valtorta F (1999) Neurite extension occurs in the absence of regulated exocytosis in PC12 subclones. Mol Biol Cell 10:2919-2931.

Leube RE (1995) The topogenic fate of the polytopic transmembrane proteins, synaptophysin and connexin, is determined by their membrane-spanning domains. J Cell Sci 108 (Pt 3):883-894.

Leube RE, Leimer U, Grund C, Franke WW, Harth N, Wiedenmann B (1994) Sorting of synaptophysin into special vesicles in nonneuroendocrine epithelial cells. J Cell Biol 127:1589-1601.

Leube RE, Wiedenmann B, Franke WW (1989) Topogenesis and sorting of synaptophysin: Synthesis of a synaptic vesicle protein from a gene transfected into nonneuroendocrine cells. Cell 59:433-446.

Leube RE, Kaiser P, Seiter A, Zimbelmann R, Franke WW, Rehm H, Knaus P, Prior P, Betz H, Reinke H (1987) Synaptophysin: Molecular organization and mRNA expression as determined from cloned cDNA. EMBO J 6:3261-3268.

Leveque C, el Far O, Martin-Moutot N, Sato K, Kato R, Takahashi M, Seagar MJ (1994) Purification of the N-type calcium channel associated with syntaxin and synaptotagmin. A complex implicated in synaptic vesicle exocytosis. J Biol Chem 269:6306-6312.

Li C, Ullrich B, Zhang JZ, Anderson RG, Brose N, Sudhof TC (1995a) Ca(2+)-dependent and -independent activities of neural and non-neural synaptotagmins. Nature 375:594-599.

Li L, Chin LS, Shupliakov O, Brodin L, Sihra TS, Hvalby O, Jensen V, Zheng D, McNamara JO, Greengard P (1995b) Impairment of synaptic vesicle clustering and of synaptic transmission, and increased seizure propensity, in synapsin I-deficient mice. Proc Natl Acad Sci U S A 92:9235-9239.

Li Z, Murthy VN (2001) Visualizing postendocytic traffic of synaptic vesicles at hippocampal synapses. Neuron 31:593-605.

Lichtenstein Y, Desnos C, Faundez V, Kelly RB, Clift-O'Grady L (1998) Vesiculation and sorting from PC12-derived endosomes in vitro. Proc Natl Acad Sci U S A 95:11223-11228.

Lin JW, Sugimori M, Llinas RR, McGuinness TL, Greengard P (1990) Effects of synapsin I and calcium/calmodulin-dependent protein kinase II on spontaneous neurotransmitter release in the squid giant synapse. Proc Natl Acad Sci U S A 87:8257-8261.

Linstedt AD, Kelly RB (1991a) Endocytosis of the synaptic vesicle protein, synaptophysin, requires the COOH-terminal tail. J Physiol (Paris) 85:90-96.

Linstedt AD, Kelly RB (1991b) Synaptophysin is sorted from endocytotic markers in neuroendocrine PC12 cells but not transfected fibroblasts. Neuron 7:309-317.

Littleton JT, Serano TL, Rubin GM, Ganetzky B, Chapman ER (1999) Synaptic function modulated by changes in the ratio of synaptotagmin I and IV. Nature 400:757-760.

Liu G, Tsien RW (1995) Properties of synaptic transmission at single hippocampal synaptic boutons. Nature 375:404-408.

Llinas R, Gruner JA, Sugimori M, McGuinness TL, Greengard P (1991) Regulation by synapsin I and ca(2+)-calmodulin-dependent protein kinase II of the transmitter release in squid giant synapse. J Physiol 436:257-282.

Llinas R, McGuinness TL, Leonard CS, Sugimori M, Greengard P (1985) Intraterminal injection of synapsin I or calcium/calmodulin-dependent protein kinase II alters neurotransmitter release at the squid giant synapse. Proc Natl Acad Sci U S A 82:3035-3039.

Lockerbie RO, Miller VE, Pfenninger KH (1991) Regulated plasmalemmal expansion in nerve growth cones. J Cell Biol 112:1215-1227.

Lonart G, Schoch S, Kaeser PS, Larkin CJ, Sudhof TC, Linden DJ (2003) Phosphorylation of RIM1alpha by PKA triggers presynaptic long-term potentiation at cerebellar parallel fiber synapses. Cell 115:49-60.

Lotti F, Menguzzato E, Rossi C, Naldini L, Ailles L, Mavilio F, Ferrari G (2002) Transcriptional targeting of lentiviral vectors by long terminal repeat enhancer replacement. J Virol 76:3996-4007.

Lu B, Czernik AJ, Popov S, Wang T, Poo MM, Greengard P (1996) Expression of synapsin I correlates with maturation of the neuromuscular synapse. Neuroscience 74:1087-1097.

Marazzi G, Buckley KM (1993) Accumulation of mRNAs encoding synaptic vesicle-specific proteins precedes neurite extension during early neuronal development. Dev Dyn 197:115-124.

Marquez-Sterling NR, Lo AC, Sisodia SS, Koo EH (1997) Trafficking of cell-surface beta-amyloid precursor protein: Evidence that a sorting intermediate participates in synaptic vesicle recycling. J Neurosci 17:140-151.

Martin TF (2000) Racing lipid rafts for synaptic-vesicle formation. Nat Cell Biol 2:E9-11.

Martina JA, Bonangelino CJ, Aguilar RC, Bonifacino JS (2001) Stonin 2: An adaptor-like protein that interacts with components of the endocytic machinery. J Cell Biol 153:1111-1120.

Martinez-Arca S, Arold S, Rudge R, Laroche F, Galli T (2004) A mutant impaired in SNARE complex dissociation identifies the plasma membrane as first target of synaptobrevin 2. Traffic 5:371-382.

Marxen M, Volknandt W, Zimmermann H (1999) Endocytic vacuoles formed following a short pulse of K+ -stimulation contain a plethora of presynaptic membrane proteins. Neuroscience 94:985-996.

Marxen M, Maienschein V, Volknandt W, Zimmermann H (1997) Immunocytochemical localization of synaptic proteins at vesicular organelles in PC12 cells. Neurochem Res 22:941-950.

Mason CA (1986) Axon development in mouse cerebellum: Embryonic axon forms and expression of synapsin I. Neuroscience 19:1319-1333.

Matteoli M, Coco S, Schenk U, Verderio C (2004) Vesicle turnover in developing neurons: How to build a presynaptic terminal. Trends Cell Biol 14:133-140.

Matteoli M, Takei K, Perin MS, Sudhof TC, De Camilli P (1992) Exo-endocytotic recycling of synaptic vesicles in developing processes of cultured hippocampal neurons. J Cell Biol 117:849-861.

McMahon HT, Bolshakov VY, Janz R, Hammer RE, Siegelbaum SA, Sudhof TC (1996) Synaptophysin, a major synaptic vesicle protein, is not essential for neurotransmitter release. Proc Natl Acad Sci U S A 93:4760-4764.

McPherson PS, Garcia EP, Slepnev VI, David C, Zhang X, Grabs D, Sossin WS, Bauerfeind R, Nemoto Y, De Camilli P (1996) A presynaptic inositol-5-phosphatase. Nature 379:353-357.

Menegon A, Verderio C, Leoni C, Benfenati F, Czernik AJ, Greengard P, Matteoli M, Valtorta F (2002) Spatial and temporal regulation of Ca2+/calmodulin-dependent protein kinase II activity in developing neurons. J Neurosci 22:7016-7026.

Ming GL, Song HJ, Berninger B, Holt CE, Tessier-Lavigne M, Poo MM (1997) cAMP-dependent growth cone guidance by netrin-1. Neuron 19:1225-1235.

Mitchell SJ, Ryan TA (2004) Syntaxin-1A is excluded from recycling synaptic vesicles at nerve terminals. J Neurosci 24:4884-4888.

Monck JR, Fernandez JM (1994) The exocytotic fusion pore and neurotransmitter release. Neuron 12:707-716.

Monck JR, Alvarez de Toledo G, Fernandez JM (1990) Tension in secretory granule membranes causes extensive membrane transfer through the exocytotic fusion pore. Proc Natl Acad Sci U S A 87:7804-7808.

Morales M, Colicos MA, Goda Y (2000) Actin-dependent regulation of neurotransmitter release at central synapses. Neuron 27:539-550.

Morgan JR, Zhao X, Womack M, Prasad K, Augustine GJ, Lafer EM (1999) A role for the clathrin assembly domain of AP180 in synaptic vesicle endocytosis. J Neurosci 19:10201-10212.

Morimoto T, Popov S, Buckley KM, Poo MM (1995) Calcium-dependent transmitter secretion from fibroblasts: Modulation by synaptotagmin I. Neuron 15:689-696.

Moulder KL, Mennerick S (2005) Reluctant vesicles contribute to the total readily releasable pool in glutamatergic hippocampal neurons. J Neurosci 25:3842-3850.

Mullany PM, Lynch MA (1998) Evidence for a role for synaptophysin in expression of long-term potentiation in rat dentate gyrus. Neuroreport 9:2489-2494.

Mundigl O, Matteoli M, Daniell L, Thomas-Reetz A, Metcalf A, Jahn R, De Camilli P (1993) Synaptic vesicle proteins and early endosomes in cultured hippocampal neurons: Differential effects of brefeldin A in axon and dendrites. J Cell Biol 122:1207-1221.

Munno DW, Prince DJ, Syed NI (2003) Synapse number and synaptic efficacy are regulated by presynaptic cAMP and protein kinase A. J Neurosci 23:4146-4155.

Murthy M, Garza D, Scheller RH, Schwarz TL (2003) Mutations in the exocyst component Sec5 disrupt neuronal membrane traffic, but neurotransmitter release persists. Neuron 37:433-447.

Murthy VN, Stevens CF (1998) Synaptic vesicles retain their identity through the endocytic cycle. Nature 392:497-501.

Nagy G, Reim K, Matti U, Brose N, Binz T, Rettig J, Neher E, Sorensen JB (2004) Regulation of releasable vesicle pool sizes by protein kinase A-dependent phosphorylation of SNAP-25. Neuron 41:417-429.

Nakata T, Terada S, Hirokawa N (1998) Visualization of the dynamics of synaptic vesicle and plasma membrane proteins in living axons. J Cell Biol 140:659-674.

Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272:263-267.

Navone F, Jahn R, Di Gioia G, Stukenbrok H, Greengard P, De Camilli P (1986) Protein p38: An integral membrane protein specific for small vesicles of neurons and neuroendocrine cells. J Cell Biol 103:2511-2527.

Neher E (1998) Vesicle pools and Ca2+ microdomains: New tools for understanding their roles in neurotransmitter release. Neuron 20:389-399.

Nestler EJ, Greengard P (1982) Nerve impulses increase the phosphorylation state of protein I in rabbit superior cervical ganglion. Nature 296:452-454.

Newman LS, McKeever MO, Okano HJ, Darnell RB (1995) Beta-NAP, a cerebellar degeneration antigen, is a neuron-specific vesicle coat protein. Cell 82:773-783.

Nichols RA, Chilcote TJ, Czernik AJ, Greengard P (1992) Synapsin I regulates glutamate release from rat brain synaptosomes. J Neurochem 58:783-785.

Nicholson-Tomishima K, Ryan TA (2004) Kinetic efficiency of endocytosis at mammalian CNS synapses requires synaptotagmin I. Proc Natl Acad Sci U S A 101:16648-16652.

Nonet ML, Holgado AM, Brewer F, Serpe CJ, Norbeck BA, Holleran J, Wei L, Hartwieg E, Jorgensen EM, Alfonso A (1999) UNC-11, a caenorhabditis elegans AP180 homologue, regulates the size and protein composition of synaptic vesicles. Mol Biol Cell 10:2343-2360.

Norcott JP, Solari R, Cutler DF (1996) Targeting of P-selectin to two regulated secretory organelles in PC12 cells. J Cell Biol 134:1229-1240.

Obendorf D, Schwarzenbrunner U, Fischer-Colbrie R, Laslop A, Winkler H (1988) In adrenal medulla synaptophysin (protein p38) is present in chromaffin granules and in a special vesicle population. J Neurochem 51:1573-1580.

Okada Y, Yamazaki H, Sekine-Aizawa Y, Hirokawa N (1995) The neuron-specific kinesin superfamily protein KIF1A is a unique monomeric motor for anterograde axonal transport of synaptic vesicle precursors. Cell 81:769-780.

Orci L, Gabbay KH, Malaisse WJ (1972) Pancreatic beta-cell web: Its possible role in insulin secretion. Science 175:1128-1130.

Osborne SL, Herreros J, Bastiaens PI, Schiavo G (1999) Calcium-dependent oligomerization of synaptotagmins I and II. synaptotagmins I and II are localized on the same synaptic vesicle and heterodimerize in the presence of calcium. J Biol Chem 274:59-66.

Paillart C, Li J, Matthews G, Sterling P (2003) Endocytosis and vesicle recycling at a ribbon synapse. J Neurosci 23:4092-4099.

Pang DT, Wang JK, Valtorta F, Benfenati F, Greengard P (1988) Protein tyrosine phosphorylation in synaptic vesicles. Proc Natl Acad Sci U S A 85:762-766.

Partoens P, Slembrouck D, Quatacker J, Baudhuin P, Courtoy PJ, De Potter WP (1998) Retrieved constituents of large dense-cored vesicles and synaptic vesicles intermix in stimulation-induced early endosomes of noradrenergic neurons. J Cell Sci 111 (Pt 6):681-689.

Parton RG, Dotti CG (1993) Cell biology of neuronal endocytosis. J Neurosci Res 36:1-9.

Parton RG, Simons K, Dotti CG (1992) Axonal and dendritic endocytic pathways in cultured neurons. J Cell Biol 119:123-137.

Pennuto M, Dunlap D, Contestabile A, Benfenati F, Valtorta F (2002) Fluorescence resonance energy transfer detection of synaptophysin I and vesicle-associated membrane protein 2 interactions during exocytosis from single live synapses. Mol Biol Cell 13:2706-2717.

Petrucci TC, Macioce P, Paggi P (1991) Axonal transport kinetics and posttranslational modification of synapsin I in mouse retinal ganglion cells. J Neurosci 11:2938-2946.

Pfrieger FW (2003) Role of cholesterol in synapse formation and function. Biochim Biophys Acta 1610:271-280.

Pieribone VA, Shupliakov O, Brodin L, Hilfiker-Rothenfluh S, Czernik AJ, Greengard P (1995) Distinct pools of synaptic vesicles in neurotransmitter release. Nature 375:493-497.

Polo-Parada L, Bose CM, Landmesser LT (2001) Alterations in transmission, vesicle dynamics, and transmitter release machinery at NCAM-deficient neuromuscular junctions. Neuron 32:815-828.

Popov SV, Poo MM (1993) Synaptotagmin: A calcium-sensitive inhibitor of exocytosis? Cell 73:1247-1249.

Poskanzer KE, Marek KW, Sweeney ST, Davis GW (2003) Synaptotagmin I is necessary for compensatory synaptic vesicle endocytosis in vivo. Nature 426:559-563.

Powelka AM, Buckley KM (2001) Expression of ARF6 mutants in neuroendocrine cells suggests a role for ARF6 in synaptic vesicle biogenesis. FEBS Lett 501:47-50.

Prekeris R, Foletti DL, Scheller RH (1999) Dynamics of tubulovesicular recycling endosomes in hippocampal neurons. J Neurosci 19:10324-10337.

Prekeris R, Klumperman J, Chen YA, Scheller RH (1998) Syntaxin 13 mediates cycling of plasma membrane proteins via tubulovesicular recycling endosomes. J Cell Biol 143:957-971.

Prekeris R, Terrian DM (1997) Brain myosin V is a synaptic vesicle-associated motor protein: Evidence for a Ca2+-dependent interaction with the synaptobrevin-synaptophysin complex. J Cell Biol 137:1589-1601.

Provoda CJ, Waring MT, Buckley KM (2000) Evidence for a primary endocytic vesicle involved in synaptic vesicle biogenesis. J Biol Chem 275:7004-7012.

Pyle JL, Kavalali ET, Piedras-Renteria ES, Tsien RW (2000) Rapid reuse of readily releasable pool vesicles at hippocampal synapses. Neuron 28:221-231.

Rahamimoff R, DeRiemer SA, Sakmann B, Stadler H, Yakir N (1988) Ion channels in synaptic vesicles from torpedo electric organ. Proc Natl Acad Sci U S A 85:5310-5314.

Regnier-Vigouroux A, Tooze SA, Huttner WB (1991) Newly synthesized synaptophysin is transported to synaptic-like microvesicles via constitutive secretory vesicles and the plasma membrane. EMBO J 10:3589-3601.

Rehm H, Wiedenmann B, Betz H (1986) Molecular characterization of synaptophysin, a major calcium-binding protein of the synaptic vesicle membrane. EMBO J 5:535-541.

Reim K, Mansour M, Varoqueaux F, McMahon HT, Sudhof TC, Brose N, Rosenmund C (2001) Complexins regulate a late step in Ca2+-dependent neurotransmitter release. Cell 104:71-81.

Reisinger C, Yelamanchili SV, Hinz B, Mitter D, Becher A, Bigalke H, Ahnert-Hilger G (2004) The synaptophysin/synaptobrevin complex dissociates independently of neuroexocytosis. J Neurochem 90:1-8.

Rigoni M, Schiavo G, Weston AE, Caccin P, Allegrini F, Pennuto M, Valtorta F, Montecucco C, Rossetto O (2004) Snake presynaptic neurotoxins with phospholipase A2 activity induce punctate swellings of neurites and exocytosis of synaptic vesicles. J Cell Sci 117:3561-3570.

Robinson MS (1994) The role of clathrin, adaptors and dynamin in endocytosis. Curr Opin Cell Biol 6:538-544.

Roos J, Kelly RB (2000) Preassembly and transport of nerve terminals: A new concept of axonal transport. Nat Neurosci 3:415-417.

Roos J, Kelly RB (1999) The endocytic machinery in nerve terminals surrounds sites of exocytosis. Curr Biol 9:1411-1414.

Rosahl TW, Spillane D, Missler M, Herz J, Selig DK, Wolff JR, Hammer RE, Malenka RC, Sudhof TC (1995) Essential functions of synapsins I and II in synaptic vesicle regulation. Nature 375:488-493.

Rosahl TW, Geppert M, Spillane D, Herz J, Hammer RE, Malenka RC, Sudhof TC (1993) Short-term synaptic plasticity is altered in mice lacking synapsin I. Cell 75:661-670.

Rubenstein JL, Greengard P, Czernik AJ (1993) Calcium-dependent serine phosphorylation of synaptophysin. Synapse 13:161-172.

Ryan TA, Li L, Chin LS, Greengard P, Smith SJ (1996) Synaptic vesicle recycling in synapsin I knock-out mice. J Cell Biol 134:1219-1227.

Sabo SL, McAllister AK (2003) Mobility and cycling of synaptic protein-containing vesicles in axonal growth cone filopodia. Nat Neurosci 6:1264-1269.

Salazar G, Craige B, Love R, Kalman D, Faundez V (2005) Vglut1 and ZnT3 co-targeting mechanisms regulate vesicular zinc stores in PC12 cells. J Cell Sci 118:1911-1921.

Salazar G, Love R, Styers ML, Werner E, Peden A, Rodriguez S, Gearing M, Wainer BH, Faundez V (2004a) AP-3-dependent mechanisms control the targeting of a chloride channel (ClC-3) in neuronal and non-neuronal cells. J Biol Chem 279:25430-25439.

Salazar G, Love R, Werner E, Doucette MM, Cheng S, Levey A, Faundez V (2004b) The zinc transporter ZnT3 interacts with AP-3 and it is preferentially targeted to a distinct synaptic vesicle subpopulation. Mol Biol Cell 15:575-587.

Salem N, Faundez V, Horng JT, Kelly RB (1998) A v-SNARE participates in synaptic vesicle formation mediated by the AP3 adaptor complex. Nat Neurosci 1:551-556.

Samigullin D, Bill CA, Coleman WL, Bykhovskaia M (2004) Regulation of transmitter release by synapsin II in mouse motor terminals. J Physiol 561:149-158.

Sampo B, Kaech S, Kunz S, Banker G (2003) Two distinct mechanisms target membrane proteins to the axonal surface. Neuron 37:611-624.

Sankaranarayanan S, Ryan TA (2001) Calcium accelerates endocytosis of vSNAREs at hippocampal synapses. Nat Neurosci 4:129-136.

Sankaranarayanan S, Ryan TA (2000) Real-time measurements of vesicle-SNARE recycling in synapses of the central nervous system. Nat Cell Biol 2:197-204.

Sara Y, Virmani T, Deak F, Liu X, Kavalali ET (2005) An isolated pool of vesicles recycles at rest and drives spontaneous neurotransmission. Neuron 45:563-573.

Schaeffer E, Alder J, Greengard P, Poo MM (1994) Synapsin IIa accelerates functional development of neuromuscular synapses. Proc Natl Acad Sci U S A 91:3882-3886.

Schenk U, Menna E, Kim T, Passafaro M, Chang S, De Camilli P, Matteoli M (2005) A novel pathway for presynaptic mitogen-activated kinase activation via AMPA receptors. J Neurosci 25:1654-1663.

Schenk U, Verderio C, Benfenati F, Matteoli M (2003) Regulated delivery of AMPA receptor subunits to the presynaptic membrane. EMBO J 22:558-568.

Schiavo G, Matteoli M, Montecucco C (2000) Neurotoxins affecting neuroexocytosis. Physiol Rev 80:717-766.

Schiebler W, Jahn R, Doucet JP, Rothlein J, Greengard P (1986) Characterization of synapsin I binding to small synaptic vesicles. J Biol Chem 261:8383-8390.

Schmid SL (1997) Clathrin-coated vesicle formation and protein sorting: An integrated process. Annu Rev Biochem 66:511-548.

Schmid SL, McNiven MA, De Camilli P (1998) Dynamin and its partners: A progress report. Curr Opin Cell Biol 10:504-512.

Schmidt A, Hannah MJ, Huttner WB (1997) Synaptic-like microvesicles of neuroendocrine cells originate from a novel compartment that is continuous with the plasma membrane and devoid of transferrin receptor. J Cell Biol 137:445-458.

Schmidt A, Wolde M, Thiele C, Fest W, Kratzin H, Podtelejnikov AV, Witke W, Huttner WB, Soling HD (1999) Endophilin I mediates synaptic vesicle formation by transfer of arachidonate to lysophosphatidic acid. Nature 401:133-141.

Schoch S, Deak F, Konigstorfer A, Mozhayeva M, Sara Y, Sudhof TC, Kavalali ET (2001) SNARE function analyzed in synaptobrevin/VAMP knockout mice. Science 294:1117-1122.

Seamon KB, Padgett W, Daly JW (1981) Forskolin: Unique diterpene activator of adenylate cyclase in membranes and in intact cells. Proc Natl Acad Sci U S A 78:3363-3367.

Sever S, Muhlberg AB, Schmid SL (1999) Impairment of dynamin's GAP domain stimulates receptor-mediated endocytosis. Nature 398:481-486.

Shi G, Faundez V, Roos J, Dell'Angelica EC, Kelly RB (1998) Neuroendocrine synaptic vesicles are formed in vitro by both clathrin-dependent and clathrin-independent pathways. J Cell Biol 143:947-955.

Shibaguchi H, Takemura K, Kan S, Kataoka Y, Kaibara M, Saito N, Taniyama K (2000) Role of synaptophysin in exocytotic release of dopamine from xenopus oocytes injected with rat brain mRNA. Cell Mol Neurobiol 20:401-408.

Shimizu H, Kawamura S, Ozaki K (2003) An essential role of Rab5 in uniformity of synaptic vesicle size. J Cell Sci 116:3583-3590.

Shimuta M, Komazaki S, Nishi M, Iino M, Nakagawara K, Takeshima H (1998) Structure and expression of mitsugumin29 gene. FEBS Lett 431:263-267.

Shupliakov O, Low P, Grabs D, Gad H, Chen H, David C, Takei K, De Camilli P, Brodin L (1997) Synaptic vesicle endocytosis impaired by disruption of dynamin-SH3 domain interactions. Science 276:259-263.

Sihra TS, Wang JK, Gorelick FS, Greengard P (1989) Translocation of synapsin I in response to depolarization of isolated nerve terminals. Proc Natl Acad Sci U S A 86:8108-8112.

Silva AJ, Rosahl TW, Chapman PF, Marowitz Z, Friedman E, Frankland PW, Cestari V, Cioffi D, Sudhof TC, Bourtchuladze R (1996) Impaired learning in mice with abnormal short-lived plasticity. Curr Biol 6:1509-1518.

Simon AM, Goodenough DA (1998) Diverse functions of vertebrate gap junctions. Trends Cell Biol 8:477-483.

Simons K, Ikonen E (1997) Functional rafts in cell membranes. Nature 387:569-572.

Simons K, van Meer G (1988) Lipid sorting in epithelial cells. Biochemistry 27:6197-6202.

Soderling TR (1999) The ca-calmodulin-dependent protein kinase cascade. Trends Biochem Sci 24:232-236.

Song H, Ming G, He Z, Lehmann M, McKerracher L, Tessier-Lavigne M, Poo M (1998) Conversion of neuronal growth cone responses from repulsion to attraction by cyclic nucleotides. Science 281:1515-1518.

Song HJ, Poo MM (1999) Signal transduction underlying growth cone guidance by diffusible factors. Curr Opin Neurobiol 9:355-363.

Song HJ, Ming GL, Poo MM (1997) cAMP-induced switching in turning direction of nerve growth cones. Nature 388:275-279.

Spiwoks-Becker I, Vollrath L, Seeliger MW, Jaissle G, Eshkind LG, Leube RE (2001) Synaptic vesicle alterations in rod photoreceptors of synaptophysin-deficient mice. Neuroscience 107:127-142.

Stenius K, Janz R, Sudhof TC, Jahn R (1995) Structure of synaptogyrin (p29) defines novel synaptic vesicle protein. J Cell Biol 131:1801-1809.

Stowell MH, Marks B, Wigge P, McMahon HT (1999) Nucleotide-dependent conformational changes in dynamin: Evidence for a mechanochemical molecular spring. Nat Cell Biol 1:27-32.

Strasser JE, Arribas M, Blagoveshchenskaya AD, Cutler DF (1999) Secretagogue-triggered transfer of membrane proteins from neuroendocrine secretory granules to synaptic-like microvesicles. Mol Biol Cell 10:2619-2630.

Subtil A, Gaidarov I, Kobylarz K, Lampson MA, Keen JH, McGraw TE (1999) Acute cholesterol depletion inhibits clathrin-coated pit budding. Proc Natl Acad Sci U S A 96:6775-6780.

Sudhof TC (2004) The synaptic vesicle cycle. Annu Rev Neurosci 27:509-547.

Sudhof TC, De Camilli P, Niemann H, Jahn R (1993) Membrane fusion machinery: Insights from synaptic proteins. Cell 75:1-4.

Sudhof TC, Lottspeich F, Greengard P, Mehl E, Jahn R (1987a) The cDNA and derived amino acid sequences for rat and human synaptophysin. Nucleic Acids Res 15:9607.

Sudhof TC, Lottspeich F, Greengard P, Mehl E, Jahn R (1987b) A synaptic vesicle protein with a novel cytoplasmic domain and four transmembrane regions. Science 238:1142-1144.

Sugita S, Janz R, Sudhof TC (1999) Synaptogyrins regulate Ca2+-dependent exocytosis in PC12 cells. J Biol Chem 274:18893-18901.

Sun YA, Poo MM (1987) Evoked release of acetylcholine from the growing embryonic neuron. Proc Natl Acad Sci U S A 84:2540-2544.

Takamori S, Riedel D, Jahn R (2000) Immunoisolation of GABA-specific synaptic vesicles defines a functionally distinct subset of synaptic vesicles. J Neurosci 20:4904-4911.

Takei K, Mundigl O, Daniell L, De Camilli P (1996) The synaptic vesicle cycle: A single vesicle budding step involving clathrin and dynamin. J Cell Biol 133:1237-1250.

Takei K, McPherson PS, Schmid SL, De Camilli P (1995a) Tubular membrane invaginations coated by dynamin rings are induced by GTP-gamma S in nerve terminals. Nature 374:186-190.

Takei Y, Harada A, Takeda S, Kobayashi K, Terada S, Noda T, Takahashi T, Hirokawa N (1995b) Synapsin I deficiency results in the structural change in the presynaptic terminals in the murine nervous system. J Cell Biol 131:1789-1800.

Tarsa L, Goda Y (2002) Synaptophysin regulates activity-dependent synapse formation in cultured hippocampal neurons. Proc Natl Acad Sci U S A 99:1012-1016.

Teng H, Wilkinson RS (2000) Clathrin-mediated endocytosis near active zones in snake motor boutons. J Neurosci 20:7986-7993.

Terada S, Tsujimoto T, Takei Y, Takahashi T, Hirokawa N (1999) Impairment of inhibitory synaptic transmission in mice lacking synapsin I. J Cell Biol 145:1039-1048.

Thakur P, Stevens DR, Sheng ZH, Rettig J (2004) Effects of PKA-mediated phosphorylation of snapin on synaptic transmission in cultured hippocampal neurons. J Neurosci 24:6476-6481.

Thiele C, Hannah MJ, Fahrenholz F, Huttner WB (2000) Cholesterol binds to synaptophysin and is required for biogenesis of synaptic vesicles. Nat Cell Biol 2:42-49.

Thoidis G, Chen P, Pushkin AV, Vallega G, Leeman SE, Fine RE, Kandror KV (1998) Two distinct populations of synaptic-like vesicles from rat brain. Proc Natl Acad Sci U S A 95:183-188.

Thomas L, Betz H (1990) Synaptophysin binds to physophilin, a putative synaptic plasma membrane protein. J Cell Biol 111:2041-2052.

Thomas L, Hartung K, Langosch D, Rehm H, Bamberg E, Franke WW, Betz H (1988) Identification of synaptophysin as a hexameric channel protein of the synaptic vesicle membrane. Science 242:1050-1053.

Tooze SA, Stinchcombe JC (1992) Biogenesis of secretory granules. Semin Cell Biol 3:357-366.

Torri Tarelli F, Bossi M, Fesce R, Greengard P, Valtorta F (1992) Synapsin I partially dissociates from synaptic vesicles during exocytosis induced by electrical stimulation. Neuron 9:1143-1153.

Torri-Tarelli F, Villa A, Valtorta F, De Camilli P, Greengard P, Ceccarelli B (1990) Redistribution of synaptophysin and synapsin I during alpha-latrotoxin-induced release of neurotransmitter at the neuromuscular junction. J Cell Biol 110:449-459.

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci U S A 76:4350-4354.

Trudeau LE, Emery DG, Haydon PG (1996) Direct modulation of the secretory machinery underlies PKA-dependent synaptic facilitation in hippocampal neurons. Neuron 17:789-797.

Tsukita S, Ishikawa H (1980) The movement of membranous organelles in axons. electron microscopic identification of anterogradely and retrogradely transported organelles. J Cell Biol 84:513-530.

Valtorta F, Leoni C (1999) Molecular mechanisms of neurite extension. Philos Trans R Soc Lond B Biol Sci 354:387-394.

Valtorta F, Pennuto M, Bonanomi D, Benfenati F (2004) Synaptophysin: Leading actor or walk-on role in synaptic vesicle exocytosis? Bioessays 26:445-453.

Valtorta F, Meldolesi J, Fesce R (2001) Synaptic vesicles: Is kissing a matter of competence? Trends Cell Biol 11:324-328.

Valtorta F, Iezzi N, Benfenati F, Lu B, Poo MM, Greengard P (1995) Accelerated structural maturation induced by synapsin I at developing neuromuscular synapses of xenopus laevis. Eur J Neurosci 7:261-270.

Valtorta F, Greengard P, Fesce R, Chieregatti E, Benfenati F (1992) Effects of the neuronal phosphoprotein synapsin I on actin polymerization. I. evidence for a phosphorylation-dependent nucleating effect. J Biol Chem 267:11281-11288.

Valtorta F, Fesce R, Grohovaz F, Haimann C, Hurlbut WP, Iezzi N, Torri Tarelli F, Villa A, Ceccarelli B (1990) Neurotransmitter release and synaptic vesicle recycling. Neuroscience 35:477-489.

Valtorta F, Jahn R, Fesce R, Greengard P, Ceccarelli B (1988) Synaptophysin (p38) at the frog neuromuscular junction: Its incorporation into the axolemma and recycling after intense quantal secretion. J Cell Biol 107:2717-2727.

van der Sluijs P, Hull M, Huber LA, Male P, Goud B, Mellman I (1992) Reversible phosphorylation--dephosphorylation determines the localization of rab4 during the cell cycle. EMBO J 11:4379-4389.

Varoqueaux F, Sigler A, Rhee JS, Brose N, Enk C, Reim K, Rosenmund C (2002) Total arrest of spontaneous and evoked synaptic transmission but normal synaptogenesis in the absence of Munc13-mediated vesicle priming. Proc Natl Acad Sci U S A 99:9037-9042.

Verderio C, Coco S, Pravettoni E, Bacci A, Matteoli M (1999) Synaptogenesis in hippocampal cultures. Cell Mol Life Sci 55:1448-1462.

Verhage M, Maia AS, Plomp JJ, Brussaard AB, Heeroma JH, Vermeer H, Toonen RF, Hammer RE, van den Berg TK, Missler M, Geuze HJ, Sudhof TC (2000) Synaptic assembly of the brain in the absence of neurotransmitter secretion. Science 287:864-869.

Vetter J, Betz H (1989) Expression of synaptophysin in the rat pheochromocytoma cell line PC12. Exp Cell Res 184:360-366.

Vitale ML, Seward EP, Trifaro JM (1995) Chromaffin cell cortical actin network dynamics control the size of the release-ready vesicle pool and the initial rate of exocytosis. Neuron 14:353-363.

Walch-Solimena C, Blasi J, Edelmann L, Chapman ER, von Mollard GF, Jahn R (1995) The t-SNAREs syntaxin 1 and SNAP-25 are present on organelles that participate in synaptic vesicle recycling. J Cell Biol 128:637-645.

Walther K, Diril MK, Jung N, Haucke V (2004) Functional dissection of the interactions of stonin 2 with the adaptor complex AP-2 and synaptotagmin. Proc Natl Acad Sci U S A 101:964-969.

Wang Y, Thiele C, Huttner WB (2000) Cholesterol is required for the formation of regulated and constitutive secretory vesicles from the trans-golgi network. Traffic 1:952-962.

Warr O, Takahashi M, Attwell D (1999) Modulation of extracellular glutamate concentration in rat brain slices by cystine-glutamate exchange. J Physiol 514 (Pt 3):783-793.

Washbourne P, Schiavo G, Montecucco C (1995) Vesicle-associated membrane protein-2 (synaptobrevin-2) forms a complex with synaptophysin. Biochem J 305 (Pt 3):721-724.

West AE, Rachael LN, Buckley KM (1997) Targeting of the synaptic vesicle protein synaptobrevin in the axon of cultured hippocampal neurons: evidence for two distinct sorting steps. J Cell Biol 139:917-927.

Wiedenmann B, Franke WW (1985) Identification and localization of synaptophysin, an integral membrane glycoprotein of mr 38,000 characteristic of presynaptic vesicles. Cell 41:1017-1028.

Wiedenmann B, Franke WW, Kuhn C, Moll R, Gould VE (1986) Synaptophysin: A marker protein for neuroendocrine cells and neoplasms. Proc Natl Acad Sci U S A 83:3500-3504.

Wilson JM, de Hoop M, Zorzi N, Toh BH, Dotti CG, Parton RG (2000) EEA1, a tethering protein of the early sorting endosome, shows a polarized distribution in hippocampal neurons, epithelial cells, and fibroblasts. Mol Biol Cell 11:2657-2671.

Windoffer R, Borchert-Stuhltrager M, Haass NK, Thomas S, Hergt M, Bulitta CJ, Leube RE (1999) Tissue expression of the vesicle protein pantophysin. Cell Tissue Res 296:499-510.

Wucherpfennig T, Wilsch-Brauninger M, Gonzalez-Gaitan M (2003) Role of drosophila Rab5 during endosomal trafficking at the synapse and evoked neurotransmitter release. J Cell Biol 161:609-624.

Xie ZP, Poo MM (1986) Initial events in the formation of neuromuscular synapse: Rapid induction of acetylcholine release from embryonic neuron. Proc Natl Acad Sci U S A 83:7069-7073.

Yao WD, Rusch J, Poo M, Wu CF (2000) Spontaneous acetylcholine secretion from developing growth cones of drosophila central neurons in culture: Effects of cAMP-pathway mutations. J Neurosci 20:2626-2637.

Ye W, Ali N, Bembenek ME, Shears SB, Lafer EM (1995) Inhibition of clathrin assembly by high affinity binding of specific inositol polyphosphates to the synapse-specific clathrin assembly protein AP-3. J Biol Chem 270:1564-1568.

Yin Y, Dayanithi G, Lemos JR (2002) Ca(2+)-regulated, neurosecretory granule channel involved in release from neurohypophysial terminals. J Physiol 539:409-418.

Young SH, Poo MM (1983) Spontaneous release of transmitter from growth cones of embryonic neurones. Nature 305:634-637.

Zakharenko S, Chang S, O'Donoghue M, Popov SV (1999) Neurotransmitter secretion along growing nerve processes: Comparison with synaptic vesicle exocytosis. J Cell Biol 144:507-518.

Zhai RG, Vardinon-Friedman H, Cases-Langhoff C, Becker B, Gundelfinger ED, Ziv NE, Garner CC (2001) Assembling the presynaptic active zone: A characterization of an active one precursor vesicle. Neuron 29:131-143.

Zhang B, Koh YH, Beckstead RB, Budnik V, Ganetzky B, Bellen HJ (1998) Synaptic vesicle size and number are regulated by a clathrin adaptor protein required for endocytosis. Neuron 21:1465-1475.

Zhang JZ, Davletov BA, Sudhof TC, Anderson RG (1994) Synaptotagmin I is a high affinity receptor for clathrin AP-2: Implications for membrane recycling. Cell 78:751-760.

Zhao C, Takita J, Tanaka Y, Setou M, Nakagawa T, Takeda S, Yang HW, Terada S, Nakata T, Takei Y, Saito M, Tsuji S, Hayashi Y, Hirokawa N (2001) Charcot-marie-tooth disease type 2A caused by mutation in a microtubule motor KIF1Bbeta. Cell 105:587-597.

Zhao H, Nonet ML (2001) A conserved mechanism of synaptogyrin localization. Mol Biol Cell 12:2275-2289.

Zhao W, Cavallaro S, Gusev P, Alkon DL (2000) Nonreceptor tyrosine protein kinase pp60c-src in spatial learning: Synapse-specific changes in its gene expression, tyrosine phosphorylation, and protein-protein interactions. Proc Natl Acad Sci U S A 97:8098-8103.

Zimmermann H, Volknandt W, Wittich B, Hausinger A (1993) Synaptic vesicle life cycle and synaptic turnover. J Physiol Paris 87:159-170.